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<p>(54) Title: TREATMENT OF CELIAC DISEASE WITH INTERLEUKIN-15 ANTAGONISTS (57) Abstract  The invention relates to the treatment of inflammatory bowel diseases, such as celiac disease with interleukin-15 (IL-15) antagonists. Preferably the antagonists are muteins of IL-15, antibodies against IL-15 or IL-15 molecules bound to chemical groups that interfere with the ability of IL-15 to effect a signal transduction through either the <math>\beta</math> or <math>\gamma</math>-subunit of the IL-15 receptor complex, but which do not interfere with IL-15 binding to IL-15R<math>\alpha</math>.</p>		

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**TREATMENT OF CELIAC DISEASE**  
**WITH INTERLEUKIN-15-ANTAGONISTS**

The invention relates to celiac disease, and in particular to the treatment of celiac disease.

Celiac disease (CD) is caused by the ingestion of gliadin in genetically predisposed individuals 1, 2 generally leading to a wide spectrum of clinical symptoms. This pathology is characterised by specific changes at the level of the small intestine with characteristic villus atrophy 1, intraepithelial lymphocytes migration 1 and production of anti-endomysium antibodies 3, 4. The latter have been shown to be a specific marker for disease, also representing a useful tool to study the incidence of the disease 4-6. Since gluten drives this disease, a simple gluten free-diet controls all the signs of this pathology. The pathogenic mechanisms leading to full-blown CD are however not yet clarified. It is generally accepted that gluten is recognised by mucosal T cells, thus initiating an immunological cascade that finally leads to the injury of the mucosa and other disease specific signs 1, 2. Consequentially, several studies on CD have focused their attention on the role of T cells 7, in particular in defying the conditions that induce T cell activation and possibly tissue damage. Despite numerous efforts, no study has provided a definitive explanation of how T cells might cause this pathology, although intraepithelial lymphocytes (IEL) have been considered as the "key players" in this disease 1. The inventors have investigated which factor(s) was driving the massive intraepithelial migration observed upon gliadin challenge. Interleukin 15 (IL-15) has recently gained a pivotal role in inducing T cell migration 8 as well as in altering the functional characteristics of the targeted T cells 9. The inventors assessed the effect of IL-15 on mucosal T cells in an *in vitro* organ culture of small intestine 10, 11. The inventors

observed that IL-15 can induce mucosal T lymphocyte migration in celiac as well as normal individuals, although some discriminatory differences were also observed. Since IL-15 has been reported to modulate the function of intestinal epithelial cells 12, the inventors studied whether IL-15 could cause, on mucosal epithelial cells of CD patients as well as on long term established human intestinal epithelial cell lines, modifications compatible with the ones driven by gliadin challenge in celiac patients. The obtained results indicated that IL-15 might have been the cardinal factor involved in the pathogenesis of CD. The key role of IL-15 was further supported by the selective over-representation of IL-15+ cells in the small intestine of untreated CD. Furthermore by using neutralising anti-IL-15 monoclonal antibody the inventors have proved that IL-15 was essentially mediating all the effects induced by gliadin challenge in an organ culture model of CD. The inventors have also provided evidence that IL-15 plays a key role in modulating intraepithelial migration. These findings indicate that IL-15 is directly involved in the initiation and maintenance of CD, providing a novel pathogenic interpretation of this disease.

Accordingly, a first aspect of the invention provides a method of treating an inflammatory bowel disease, such as celiac disease, by administration of an antagonist of IL-15 to a patient.

A further aspect of the invention provides the use of an antagonist of IL-15 to treat an inflammatory bowel disease, such as celiac disease.

A still further aspect of the invention provides an antagonist of IL-15 for use in the manufacture of a medicament to treat an inflammatory bowel disease, such as celiac disease.

Preferably, the antagonist of IL-15 activity interferes with the signal transduction of IL-15 through its receptor complex. In particular, the IL-15 antagonists used in the invention are preferably selected from the group consisting of (a) a mutein of mature, or native, IL-15 capable of binding to the  $\alpha$ -subunit of the IL-15 receptor and incapable of transducing a signal through the  $\beta$  and/or  $\gamma$ -subunits of the IL-15 receptor complex; (b) a monoclonal antibody against IL-15 that prevents IL-15 from effecting signal transduction through the  $\beta$  and/or  $\gamma$ -subunits of the IL-15 receptor complex; and (c) an IL-15 molecule that is covalently bonded with a chemical group that interferes with the ability of IL-15 to effect a signal transduction through either the  $\beta$  or  $\gamma$ -subunits of the IL-15 receptor complex, but does not interfere with IL-15 binding to IL-15Ra. Antagonists for use in the invention also include monoclonal antibodies against IL-15.

Preferably, the antagonist used is selected from mature, or native, simian IL-15 molecules having the sequence of amino acids 49-162 of SEQ ID NO:1 or human IL-15 molecules having the sequence of amino acids 49-162 of SEQ ID NO:2, that have been mutated in order to produce an antagonist of IL-15. Such IL-15 muteins are capable of binding to the IL-15Ra subunit, and are incapable of transducing a signal through the  $\beta$  or  $\gamma$ -subunits of the IL-15 receptor complex. These are the subject of patent application number WO 96/26274.

Preferably the antagonist is supplied in a pharmaceutically effective amount. That is, an amount sufficient to reduce or remove the clinical symptoms of the inflammatory disease.

### L-15 Muteins

There are many possible mutations of IL-15 that can produce antagonists. Such mutations can be made at specific amino acid sites believed to be responsible for  $\beta$  or  $\gamma$ -subunit signalling; or mutations can be made over entire regions of IL-15 that are considered necessary for  $\beta$ - or  $\gamma$ -subunit signalling. Typically, mutations may be made as additions, substitutions or deletions of amino acid residues. Preferably, substitution and deletion muteins are preferred with substitution muteins being most preferred.

It is believed that the Asp56 affects binding with the  $\beta$ -subunit and that the Gln156 affects binding with the  $\gamma$ -subunit of the IL-15 receptor complex. Adding or substituting other naturally-occurring amino acid residues near or at sites Asp56 and Gln156 can affect the binding of IL-15 to either or both of the  $\beta$  or  $\gamma$ -subunits of the IL-15 receptor complex. Indeed, removing the negatively-charged aspartic acid residue and replacing it with another negatively-charged residue may not be as effective at blocking receptor binding as if the aspartic acid were replaced with a positively-charged amino acid such as arginine, or uncharged residues such as serine or cysteine.

Recombinant production of an IL-15 mutein first requires isolation of a DNA clone (i.e., cDNA) that encodes an IL-15 mutein. cDNA clones are derived from primary cells or cell lines that express mammalian IL-15 polypeptides. First total cell mRNA is isolated, then a cDNA library is made from the mRNA by reverse transcription. A cDNA clone may be

isolated and identified using the DNA sequence information provided herein to design a cross-species hybridization probe or PCR primer as described above. Such cDNA clones may have the sequence of nucleic acids 1-489 of SEQ ID NO:3 and SEQ ID NO:4.

The isolated cDNA is preferably in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns. Genomic DNA containing the relevant nucleotide sequences that encode mammalian IL-15 polypeptides can also be used as a source of genetic information useful in constructing coding sequences. The isolated cDNA can be mutated utilising techniques known in the art to provide IL-15 antagonist activity.

Equivalent DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for activity are encompassed by the invention. For example, N-glycosylation sites in IL-15 can be modified to preclude glycosylation, allowing expression of a reduced carbohydrate analog in mammalian and yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterised by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. The simian IL-15 protein comprises two such triplets, at amino acids 127-129 and 160-162 of SEQ ID NO:1. The human IL-15 protein comprises three such triplets, at amino acids 119-121, 127-129 and 160-162 of SEQ ID NO:2. Appropriate substitutions, additions or deletions to the nucleotide sequence encoding these triplets will result in prevention of attachment of carbohydrate residues at the Asn side chain. Alteration of a single nucleotide, chosen so that Asn is replaced by a different amino acid, for example, is sufficient to inactivate an N-glycosylation site.



Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846, hereby incorporated by reference.

Recombinant expression vectors include synthetic or cDNA-derived DNA fragments encoding an IL-15 mutein. The DNA encoding an IL-15 mutein is operably linked to a suitable transcriptional or translational regulatory or structural nucleotide sequence, such as one derived from mammalian, microbial, viral or insect genes. Examples of regulatory sequences include, for example, a genetic sequence having a regulatory role in gene expression (e.g., transcriptional promoters or enhancers), an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and appropriate sequences that control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the structural gene. For example, a DNA sequence for a signal peptide (secretory leader) may be operably linked to a structural gene DNA sequence for an IL-15 mutein, the signal peptide is expressed as part of a precursor amino acid sequence and participates in the secretion of an IL-15 mutein. Further, a promoter nucleotide sequence is operably linked to a coding sequence (e.g., structural gene DNA) if the promoter nucleotide sequence controls the transcription of the structural gene nucleotide sequence. Still further, a ribosome binding site may be operably linked to a structural gene nucleotide coding sequence (e.g. IL-15 mutein) if the ribosome binding site is positioned within the vector to encourage translation.

Suitable host cells for expression of an IL-15 mutein include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram



negative or gram positive organisms. for example *E. coli* or bacilli. Suitable prokaryotic hosts cells for transformation include, for example, *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. As discussed in greater detail below, examples of suitable host cells also include yeast such as *S. cerevisiae*, a mammalian cell line such as Chinese Hamster Ovary (CHO) cells, or insect cells. Cell-free translation systems could also be employed to produce an IL-15 mutein using RNAs derived from the DNA constructs disclosed herein. Appropriate cloning and expression vectors for use with bacterial, insect, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. Cloning Vectors: A Laboratory Manual. Elsevier, New York. 1985.

When an IL-15 mutein is expressed in a yeast host cell, the nucleotide sequence (e.g., structural gene) that encodes an IL-15 mutein may include a leader sequence. The leader sequence may enable improved extracellular secretion of translated polypeptide by a yeast host cell.

IL-15 muteins may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2 $\mu$  yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, and sequences for transcription termination. Preferably, yeast vectors include an origin of replication sequence and selectable marker. Suitable promoter sequences for yeast vectors include promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255:2073, 1980) or other

glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7:149, 1968; and Holland et al., Biochem. 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EP-A-73,657.

Yeast vectors can be assembled, for example, using DNA sequences from pBR322 for selection and replication in *E. coli* (Ampr gene and origin of replication). Other yeast DNA sequences that can be included in a yeast expression construct include a glucose-repressible ADH2 promoter and a-factor secretion leader. The ADH2 promoter has been described by Russell et al. (J. Biol. Chem. 258:2674, 1982) and Beier et al. (Nature 300:724, 1982). The yeast *a*-factor leader sequence directs secretion of heterologous polypeptides. The *a*-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. See, e.g., Kurjan et al., Cell 30:933, 1982; and Bitter et al., Proc. Natl. Acad. Sci. USA 81:5330, 1984. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those skilled in the art. One such protocol is described by Hinnen et al., Proc. Natl. Acad. Sci. USA 75:1929, 1978. The Hinnen et al. protocol selects for Trp<sup>+</sup> transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 mg/ml adenine and 20 mg/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 mg/ml adenine and 80 mg/ml uracil. Repression of the ADH2 promoter is lost when glucose is exhausted from the medium.

Alternatively, in a prokaryotic host cell, such as *E. coli*, the IL-15 mutein may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in a prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant IL-15 mutein.

The recombinant expression vectors carrying the recombinant IL-15 mutein structural gene nucleotide sequence are transfected or transformed into a suitable host microorganism or mammalian cell line.

Expression vectors transfected into prokaryotic host cells generally comprise one or more phenotypic selectable markers. A phenotypic selectable marker is, for example, a gene encoding proteins that confer antibiotic resistance or that supply an autotrophic requirement, and an origin of replication recognised by the host to ensure amplification within the host. Other useful expression vectors for prokaryotic host cells include a selectable marker of bacterial origin derived from commercially available plasmids. This selectable marker can comprise genetic elements of the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus

provides simple means for identifying transformed cells. The pBR322 "backbone" sections are combined with an appropriate promoter and a IL-15 mutein structural gene sequence. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences are commonly used for recombinant prokaryotic host cell expression vectors. Common promoter sequences include  $\beta$ -lactamase (penicillinase), lactose promoter system (Chang et al., Nature 275:615, 1978; and Goeddel et al., Nature 281:544, 1979), tryptophan (trp) promoter system (Goeddel et al., Nucl. Acids Res. 8:4057, 1980; and EPA 36.776) and tac promoter (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, (1989)). A particularly useful prokaryotic host cell expression system employs a phage  $\lambda$  PL promoter and a  $\text{cl}857\text{ts}$  thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection that incorporate derivatives of the  $\lambda$  PL promoter include plasmid pHUB2 (resident in E. coli strain JMB9 (ATCC 37092)) and pPLc28 (resident in E. coli RR1 (ATCC 53082)).

Mammalian or insect host cell culture systems also could be employed to express recombinant IL-15 muteins. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells (Gluzman et al., Cell 23:175, (1981); ATCC CRL 1651), L cells, C127 cells, 3T3 cells (ATCC CCL 163), CHO cells, HeLa cells (ATCC CCL 2), and BHK (ATCC CRL 10) cell lines. Suitable mammalian expression vectors include nontranscribed elements such as an origin of replication, a promoter sequence, an enhancer linked to the structural gene, other 5' or 3' flanking nontranscribed sequences.

such as ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

Transcriptional and translational control sequences in mammalian host cell expression vectors may be provided by viral sources. For example, commonly used mammalian cell promoter sequences and enhancer sequences are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment that may also contain a viral origin of replication (Fiers et al., Nature 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the SV40 viral origin of replication site is included.

Exemplary mammalian expression vectors can be constructed as disclosed by Okayama and Berg (Mol. Cell. Biol. 3:280, 1983). Additional useful mammalian expression vectors are described in U.S. Patent Application Serial No. 07/480,694 filed February 14, 1990 and U.S. Patent 5,350,683.

#### **Purification of Recombinant IL-15 Muteins**

In general, IL-15 mutein polypeptides may be prepared by culturing transformed host cells under culture conditions necessary to express IL-15 mutein polypeptides. The resulting

expressed mutein may then be purified from culture media or cell extracts. An IL-15 mutein may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. With or without the concentration step, the culture media can be applied to a purification matrix such as a hydrophobic chromatography medium. Phenyl Sepharose<sup>®</sup> CL-4B (Pharmacia) is the preferred medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, gel filtration medium can be used.

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant butyl or other aliphatic groups, can be employed to further purify IL-15 muteins. An S Sepharose (Pharmacia) cation exchange column may also be employed as a final buffer exchange step. Some or all of the foregoing conventional purification steps, in various combinations, can also be employed to provide a substantially homogeneous recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any



convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells are preferably employed to express an IL-15 mutein as a secreted polypeptide. Secreted recombinant polypeptide from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (J. Chromatog. 296:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

Preferably, a mutein of IL-15 is used wherein at least one of the amino acid residues Asp56 or Gln156 of IL-15 (simian IL-15 having the sequence of amino acid residues 49-162 shown in SEQ ID NO:1 or human IL-15 having the sequence of amino acid residues 49-162 shown in SEQ ID NO:2) is deleted or substituted with a different naturally-occurring amino acid residue. Any combination of substitutions and/or deletions can be made. For example, Asp56 can be deleted while Gln156 is substituted with any other amino acid, or both Asp56 and Gln156 are each substituted with the same or different amino acid moiety. Further, Asp56 can be substituted with any amino acid while Gln156 is deleted. Generally, substitution muteins are preferred, and more preferred are those that do not severely affect the natural folding of the IL-15 molecule. Substitution muteins preferably include those wherein Asp56 is substituted by serine or cysteine; or wherein Gln156 is substituted by serine or cysteine, or wherein both Asp56 and Gln156 are each substituted with a serine or cysteine. Examples of deletion muteins include those wherein Asp56 and Gln156 of mature IL-15 are both deleted; wherein only Asp56 is deleted; or wherein only Gln156 is deleted. It is possible that other amino acid residues in



the region of either Asp56 and Gln156 can be substituted or deleted and still have an effect of preventing signal transduction through either or both of the  $\beta$  or  $\gamma$ -subunits of the IL-15 receptor complex. Therefore, the invention further encompasses muteins wherein amino acid residues within the region of Asp56 and Gln156 are either substituted or deleted, and that possess IL-15 antagonist activity. Such muteins can be made using the methods described herein and can be assayed for IL-15 antagonist activity using conventional methods.

### **Conjugated IL-15 Molecules and IL-15 Muteins**

The mature IL-15 polypeptides disclosed herein (mature simian IL-15 comprising the sequence of amino acids 49-162 of SEQ ID NO:1 and mature human IL-15 having the sequence of amino acid residues 49-162 shown in SEQ ID NO:2), as well as the IL-15 muteins, may be modified by forming covalent or aggregative conjugates with other chemical moieties. Such moieties can include PEG, mPEG, dextran, PVP, PVA, polyamino acids such as poly-L-lysine or polyhistidine, albumin and gelatin at specific sites on the IL-15 molecule that can interfere with binding of IL-15 to the  $\beta$  or  $\gamma$ -chains of the IL-15 receptor complex, while maintaining the high affinity of IL-15 for the IL-15R $\alpha$ . Additionally, IL-15 can be specifically glycosylated at sites that can interfere with binding of IL-15 to the  $\beta$  or  $\gamma$ -chains of the IL-15 receptor complex, while maintaining the high affinity of IL-15 for the IL-15R $\alpha$ . Preferred groups for conjugation are PEG, dextran and PVP. Most preferred for use in the invention is PEG, wherein the molecular weight of the PEG is preferably between about 1,000 to about 20,000. A molecular weight of about 5000 is preferred for use in conjugating IL-15, although PEG molecules of other weights would be suitable as well. A variety of forms of PEG are suitable for use in the invention.

For example, PEG can be used in the form of succinimidyl succinate PEG (SS-PEG) which provides an ester linkage that is susceptible to hydrolytic cleavage in vivo, succinimidyl carbonate PEG (SC-PEG) which provides a urethane linkage and is stable against hydrolytic cleavage in vivo, succinimidyl propionate PEG (SPA-PEG) provides an ether bond that is stable in vivo, vinyl sulfone PEG (VS-PEG) and maleimide PEG (Mal-PEG) all of which are commercially available from Shearwater Polymers, Inc. (Huntsville, AL). In general, SS-PEG, SC-PEG and SPA-PEG react specifically with lysine residues in the polypeptide, whereas VS-PEG and Mal-PEG each react with free cysteine residues. However, Mal-PEG is prone to react with lysine residues at alkaline pH. Preferably, SC-PEG and VS-PEG are preferred, and SC-PEG is most preferred due to its in vivo stability and specificity for lysine residues.

The PEG moieties can be bonded to IL-15 in strategic sites to take advantage of PEGOs large molecular size. As described above, PEG moieties can be bonded to IL-15 by utilising lysine or cysteine residues naturally occurring in the protein or by site-specific PEGylation. One method of site specific PEGylation is through methods of protein engineering wherein cysteine or lysine residues are introduced into IL-15 at specific amino acid locations. The large molecular size of the PEG chain(s) conjugated to IL-15 is believed to block the region of IL-15 that binds to the  $\beta$  and/or  $\gamma$ -subunits but not the  $\alpha$ -subunit of the IL-15 receptor complex. Conjugations can be made by a simple addition reaction wherein PEG is added to a basic solution containing IL-15. Typically, PEGylation is carried out at either (1) about pH 9.0 and at molar ratios of SC-PEG to lysine residue of approximately 1:1 to 100:1, or greater; or (2) at about pH 7.0 and at molar ratios of VS-PEG to cysteine residue of approximately 1:1 to 100:1, or greater.

Characterization of the conjugated PEGylated IL-15 molecules can be performed by SDS-PAGE on a 4-20 % gradient polyacrylamide gel, available from Novex Corp., San Diego, California. Conventional silver staining may be employed, or conventional Western blotting techniques can be utilised for highly PEGylated proteins that are not visualised easily by silver staining. Purification of the PEGylated IL-15 molecules can be performed using size exclusion chromatography, dialysis, ultrafiltration or affinity purification.

The extent of modification and heterogeneity of PEGylated IL-15 can be determined as eg conventional matrix assisted laser desorption ionization mass spectrometry (MALDI). Since human IL-15 has a molecular weight of about 13,000 and by using PEG having a molecular weight of 5000, MALDI indicates that preparations weighing 13,000 are unPEGylated, those weighing 18,000 indicate that 1 molecule of IL-15 is bonded to one PEG molecule; those weighing 23,000 signify that one IL-15 molecule is bound with two PEG molecules, etc.

#### **Monoclonal Antibodies Against IL-15**

Alternatively, an antagonist according to the invention can take the form of a monoclonal antibody against IL-15 that interferes with the binding of IL-15 to any of the  $\alpha$ ,  $\beta$  or  $\gamma$ -subunits of the IL-15 receptor complex. Within one aspect of the invention, IL-15, including derivatives thereof, as well as portions or fragments of these proteins such as IL-15 peptides, can be used to prepare antibodies that specifically bind to IL-15. Within the context of the invention, the term "antibodies" should be understood to include

polyclonal antibodies, monoclonal antibodies, fragments thereof such as F(ab')<sub>2</sub> and Fab fragments, as well as recombinantly produced binding partners. The affinity of a monoclonal antibody or binding partner may be readily determined by one of ordinary skill in the art (see Scatchard, Ann. N.Y. Acad. Sci., 51: 660-672 (1949)).

In general, monoclonal antibodies against IL-15 can be generated using the following procedure. Purified IL-15, a fragment thereof, synthetic peptides or cells that express IL-15 can be used to generate monoclonal antibodies against IL-15 using techniques known per se, for example, those techniques described in U.S. Patent 4,411,993. Briefly, mice are immunised with IL-15 as an immunogen emulsified in complete Freund's adjuvant or RIBI adjuvant (RIBI Corp., Hamilton, Montana), and injected in amounts ranging from 10-100  $\mu$ g subcutaneously or intraperitoneally. Ten to twelve days later, the immunised animals are boosted with additional IL-15 emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunisation schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision to test for IL-15 antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of IL-15 activity on CTLL-2 cells.

Following detection of an appropriate antibody titer, positive animals are provided an additional intravenous injection of IL-15 in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine,

aminopterin and thymidine) selective medium to inhibit proliferation of non-fused myeloma cells and myeloma hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified IL-15 by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990). Positive hybridoma cells can be injected intraperitoneally into syngeneic Balb/c mice to produce ascites containing high concentrations of anti-IL-15 monoclonal antibodies. Alternatively, hybridoma cells can be grown in vitro in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to IL-15.

Other "antibodies" can be prepared utilising the disclosure provided herein, and thus fall within the scope of the invention. Procedures used to generate humanized antibodies can be found in U.S. Patent No. 4,816,567 and WO 94/10332; procedures to generate microbodies can be found in WO 94/09817; and procedures to generate transgenic antibodies can be found in GB 2 272 440, all of which are incorporated herein by reference.

To determine which monoclonal antibodies are antagonists, use of a screening assay is preferred. A CTLL-2 proliferation assay is preferred for this purpose. See, Gillis and Smith, Nature 268:154 (1977), which is incorporated herein by reference.

Preferably the IL-15 antagonists are formulated according to known methods used to prepare pharmaceutically useful compositions. An IL-15 antagonist can be combined in admixture, either as the sole active material or with other known active materials, with pharmaceutically suitable diluents (e.g., Tris-HCl, acetate, phosphate), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed. 1980, Mack Publishing Co. In addition, such compositions can contain an IL-15 antagonist complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance of an IL-15 antagonist. An IL-15 antagonist can also be conjugated to antibodies against tissue-specific receptors, ligands or antigens, or coupled to ligands of tissue-specific receptors.

The IL-15 antagonist of the invention can be administered topically, orally, parenterally, rectally or by inhalation. The term "parenteral" includes subcutaneous injections, intravenous, intramuscular, intracisternal injection, or infusion techniques. These compositions will typically contain an effective amount of an IL-15 antagonist, alone or in

combination with an effective amount of any other active material. Such dosages and desired drug concentrations contained in the compositions may vary depending upon many factors, including the intended use, patient's body weight and age, and route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration can be performed according to art-accepted practices.

**Figure 1:** Effect on T lymphocyte migration of IL-15 in *in vitro* cultured treated CD (A) and control (B) intestine.

*A: Treated CD intestine:*

Migration to the subepithelial (SE) compartment of the lamina propria, number of positive cells per mm<sup>2</sup> of lamina propria, mean + SD and migration into the IEC, number of positive cells per mm epithelium, mean + SD.

•p < 0.01 and •• p<0.05 vs cultures with medium alone.

*B: Control intestine:*

Migration to the subepithelial (SE) compartment of the lamina propria and to the intraepithelial (IE) compartment.

Migration to SE, number of positive cells per mm<sup>2</sup> of lamina propria, and migration to the IE, number of positive cells per mm epithelium, mean + SD.

••<0.05 vs cultures with medium alone.



**Figure 2:** Migration of CD3+ cells to the upper mucosal layers in treated CD intestine after incubation with medium alone (A) or with IL-15 (B).

Note the massive migration of CD3+ cells to the subepithelial compartment of the lamina propria and the infiltration of the surface epithelium after IL-15 treatment (B). Only a few intraepithelial lymphocytes are found within the surface epithelium after incubation with medium alone (A).

{Original magnification x 160 (A), 180(B)}

**Figure 3:** Effect on T cell activation of IL-15 in *in vitro* cultured treated CD and control intestine

*A: Treated CD intestine*

% of CD3+ cells per mm<sup>2</sup> of lamina propria, mean + SD.

• p< 0.01 and •• p<0.05 vs cultures with medium alone

*B: Control intestine*

% of CD3+ cells per mm<sup>2</sup> of lamina propria, mean + SD.

•• p<0.05 vs cultures with medium alone.

**Figure 4:** Expression of FAS by enterocytes in treated CD intestine after challenge with medium alone (A) or with IL-15 (B).

Very low but negligible staining is observed after culture with medium alone(A). Intense FAS expression by enterocytes is detected after culture with IL-15. The staining is mainly

detected on the basolateral membranes and in the basal cytoplasm (B). *{Original magnification, x 160}*

**Figure 5:** Effect of anti-IL-15 MoAb on treated CD intestine: modulation of the expression of FAS-L by enterocytes after 24 h of organ culture in the presence of gliadin digest.

Intense expression of FAS-L is detected on cellular membranes and the whole cytoplasm after incubation with gliadin. The staining is also detected in some lamina propria cells. The same pattern is observed in this case after incubation with the sole medium or with IL-15 (A). A marked reduction of FAS-L expression is observed after treatment of biopsies challenged with gliadin added with anti-IL-15 MoAb M110 (B) *{Original magnification, x180}*.

**Figure 6:** Effect of anti-IL-15 MoAb on untreated CD intestine with villus atrophy: inhibition of enterocyte DNA fragmentation induced by 24 h organ culture in the presence of gliadin digest.

Many enterocytes are TUNEL+ after challenge with gliadin (A) ; a dramatic decrease in the number of TUNEL+ enterocytes is observed after incubation with gliadin added with M110 anti-IL-15 MoAb (B) *(Original magnification, x180)*

**Figure 7:** Effect of IL-15 and IL-2 on the expression of immunological markers after 24 h of incubation in CACO-2 cell line.

A-B: Expression of Ki67 antigen: intense expression of Ki67 is detected in almost all cells after incubation with IL-15 (A) and IL-2 (B).

C-D: Expression of FAS: intense expression of FAS is detected on the cell surface of almost all cells after IL-15 treatment (C), whereas weak but negligible staining is detected after incubation with IL-2 (D), as well as with medium alone.

E-F: Expression of FAS-Ligand: intense staining is detected on the cell surface of almost all cells after IL-15 treatment (E), whereas a weak staining is detected only in a few cells after IL-2 treatment (F), as well as with medium alone.

*{Original magnification, x180 (A,B), x240 (C,D,E,F)}.*

**Figure 8:** Effect of IL-15 on cell death in CACO-2 cell line simultaneously cultured with IL-15 vs medium alone, or vs IL-7, IL-2, gliadin, IL-15+M3MoAb: Trypan-Blue+ cells in culture supernatants.

Number of Trypan-Blue+ cells  $\times 2 \times 10^4$ /ml culture medium

Cultures with IL-15 vs cultures with: medium alone(n=7); IL-7(n=7); IL-2(n=7); gliadin(n=7); IL-15+M3 MoAb(n=5)

••.IL-15 vs medium alone, IL-7,IL-2,gliadin,IL-15+M3 MoAb. respectively , $p < 0.05$

**Figure 9:** Effect of IL-15 on CACO-2 cell line: induction of apoptosis after 24 h of incubation with IL-15.

After 24 h of IL-15 treatment, incubation with FITC Annexin-V (green colour), and propidium iodide (orange colour) leads some cells to show green colour and others to co-express nuclear orange colour (A); note green staining on cell surface together orange colour in the nucleus and in the cytoplasm in some cells (B).

*(Original magnification, x. 180 (A), x280 (B))*

## METHODS

### 1. Patients and organ tissue cultures of duodenal explants

**Patients.** 14 untreated CD patients with villus atrophy and crypt hyperplasia (mean age 44.5, range 18-60), 14 treated CD patients on gluten-free diet for at least 12 months and 8 non-CD controls (mean age 38.5, range 19-53) underwent duodenal endoscopy for diagnostic purposes. Informed consent was obtained from all patients. All treated CD patients showed normal mucosal histology, with V/C ratio  $> 3$  and absence of serum EMA, although EMA were detected in one of these treated patients in small intestine organ culture challenged for 24 hours with medium alone. Non-CD controls were affected by oesophagitis (3/8), gastritis (2/8) and chronic non-specific diarrhoea (3/8). They were all EMA negative and showed normal villus length. Intestinal specimens were obtained at the duodenal-jejunal flexure by peroral biopsy from all patients. All specimens were washed in 0.15 M sodium chloride and examined with a dissecting microscope. One specimen from each patient was oriented and embedded in optimal cutting temperature, OCT compound (Tissue Tek, Miles Laboratories, Elkhart, IN, USA), snap frozen in isopentane cooled in liquid nitrogen and then stored at  $-70^{\circ}\text{C}$  until cryosectioning.  $5\text{ }\mu\text{m}$  sections were stained with hematoxylin and used for diagnosis.

**Preparation of the culture medium and mucosal tissue culture.** Duodenal explants from treated CD patients, from 4 untreated CD with villus atrophy and 5 controls were cultured *in vitro* for 24 hours as previously reported<sup>11</sup>. IL-15 and IL-7 were obtained from Immunex (Seattle, USA), IL-2 from Roche (Basel, Switzerland), and IL-4 from Sandoz, Basel, Switzerland. Anti-IL-15 MoAbs M110 and M111 were obtained from Immunex. IL-15, IL-7, IL-2 and IL-4 were added to the medium at the final concentration of 10 ng/ml. In 4 treated CD samples, just before the *in vitro* culture, anti-IL-15 MoAbs (M110 or M111) were added at the final concentration of 5  $\mu$ g/ml to the medium containing IL-15. In 3 samples taken from treated CD patients and in 4 samples taken from untreated CD with villus atrophy, just before the *in vitro* culture, M110 MoAb was added to the medium containing PT digest, and carefully mixed to reach a final concentration of 5  $\mu$ g/ml. Anti-human lactase MoAb mlac1<sup>16</sup> was used as isotype control antibody. Duodenal explants from non-CD controls were cultured in the presence of the sole medium or of medium added with PT gliadin digest (1 mg/ml) or medium added with IL-15 (10 mg/ml).

**Immunohistochemistry.** Cryostat sections (5  $\mu$ m) were individually tested with MoAbs to different immunological markers {ICAM-1 (Dako, Copenhagen, Denmark, 1:400), CD25 (Dako, 1:30), CD3 (Dako, 1:200), CD8 (Dako, 1:200),  $\gamma/\delta$  (T Cell Diagnostics, Inc. Cambridge, MA, 1:25), Ki67 (Dako 1:25)} and immunostaining was done following the alkaline phosphatase/anti-alkaline phosphatase or peroxidase staining techniques<sup>11</sup>. MoAbs M3 and M38 and M38 FAS specific<sup>32</sup> (Immunex, mouse MoAbs, 1:30) and FAS-L specific (Alexis Bingham, UK, rat biotinylated MoAb 804-009B-T100, 1:50)<sup>33</sup> were also

used and detected by peroxidase staining technique. Intraepithelial lymphocytes stained by anti-CD3 or CD8 or  $\gamma/\delta$  MoAbs were numbered per mm epithelium; the number of stained cells in the lamina propria was calculated per mm<sup>2</sup> of lamina propria as previously reported and referred as percentage of CD3+ cells within the same mucosal area<sup>11</sup>. The number of dividing cells expressing Ki67 in crypts was calculated as previously described as percentage of crypt enterocytes<sup>34</sup>. Staining of epithelial cells by anti-FAS or anti-FAS-L was arbitrarily graded from 0 to +2. This evaluation was based on the intensity of staining of the cells [undetectable (0), low (1+), intense (2+)]. Guidelines for this scoring system were established at the start of the study and the samples were independently analysed by two observers; the results were compared afterwards. At least 5 slides for each sample were blindly evaluated for all tested markers. Two colours immunohistochemistry for the characterisation of mononuclear cell populations was performed as previously reported<sup>34</sup>. For control of specificity of the immunohistochemical data we have performed: i) incubation with mouse IgG or IgM directed against inappropriate blood group antigens and detected by peroxidase-conjugated streptavidin, as well as by peroxidase anti-peroxidase (PAP) staining technique; ii) incubation with Rat Ig MoAb against IL-2; iii) omission of primary antibody; iv) repeated experiments (three times each) in the presence of already tested positive and negative samples as internal experimental controls; v) parallel analysis of samples cultured in the presence of medium or gliadin or tested cytokines in the same experimental conditions (each subject provides an internal control).

**Antiendomysial antibodies (EMA) detection in culture supernatants.** EMA detection was sought for in undiluted culture supernatants by immunofluorescence (Eurospital Pharma, Trieste, Italy) according to experimental procedure previously reported<sup>15</sup>. The

results were blindly evaluated by two distinct observers. In cultured samples positivity was weak but clear and only detectable in undiluted supernatants and always blindly compared to the other samples belonging to the same patient.

## 2. Cell culture.

Cell culture chemicals were obtained from GIBCO-Life Technologies (Milan, Italy). Human intestinal cells CACO-2 were purchased from the Istituto Zooprofilattico della Lombardia e dell'Emilia (Brescia, Italy) and used at passage 25-40. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/L glucose and supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 2mmol/L L-glutamine, 1% penicillin-streptomycin, 1% sodium pyruvate. Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. Single cell suspensions were obtained from 70-80% confluent cultures by incubation with 0.05% trypsin and then 10<sup>5</sup> cells were seeded in 60x15 mm Petri dishes containing 20x20 mm glass coverslips.

### **Detection of cell death in culture supernatants**

One ml culture supernatant was centrifuged (10.000 rpm for 5 min) in a centrifuge Eppendorf 5415c (Hamburg, FRG). The pellet was re-suspended in 20 µl medium to which 20 µl of Trypan Blue (Sigma Chemical Co. St Louis, MO) were added. After short vortexing cells were loaded in a Neubauer chamber (Carlo Erba, Milan, Italy) and counted.

### **Annexin-V test and TUNEL.**



Detection of DNA fragmentation on cryostat tissue samples was performed by deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labelling (TUNEL) method and visualised by peroxidase staining as previously described<sup>34</sup>.

ApoAlert™ Annexin V Apoptosis Kit (Clonotech Laboratories, Inc., Palo Alto, CA) was used for detection of apoptosis in cultured CACO-2 cells. The experiments were carried out according to the manufacturer's recommendations. Briefly, coverslips were rapidly rinsed in phosphate buffer (PBS) pH 7.4, and incubated for 15 min with 200  $\mu$ l lx binding buffer. Then, 10  $\mu$ l enhanced Annexin V-FITC (final concentration 0.5  $\mu$ g/ml) and 10  $\mu$ l propidium iodide were added and incubation took place for 10 min in the dark. Coverslips were mounted and analysed under fluorescence microscope using a dual filter set for FITC & rhodamine.

### **Immunocytochemistry**

Coverslips with cells were fixed in acetone for 5 min and air dried. They were then rinsed in Tris buffered saline pH 7.36 and incubated with non-immune goat serum (1:200, DAKO) for 15 min. This followed by 1 h incubation with primary MoAb (anti-FAS, FAS-L, Ki67, as described for immunohistochemistry on tissue sections) and then by link antibody goat anti-mouse biotinylated Ig (1:300, Dako) only after anti-FAS and anti-Ki67 Ab incubation and finally by FITC streptavidin (1:50, DAKO) after all antibody incubation. Each step was followed by careful washing with TRIS saline buffer. The coverslips were then mounted and observed at optical microscope equipped with fluorescence.

### 3. Statistical analysis.

The samples belonging to each category were compared to each other. Student's two-tailed t test was used to compare intestinal samples for the expression of immunological markers in the lamina propria, for calculation of stained intraepithelial lymphocytes<sup>11</sup> and for Ki67 antigen expression<sup>34</sup>. Non parametric tests (Wilcoxon two-tailed) were also applied and the results were concordant with those obtained using parametric tests. For FAS, FAS-L and TFR expression samples with undetectable or low staining have been pooled for analysis and Fisher's test was applied to compare tissues with undetectable or low (0 to 1-) staining with those showing intense (2+) staining. For the analysis of EMA in culture supernatants Fisher's test was applied as well. For the analysis of Trypan-Blue+ cells in culture supernatants, paired t test was used to compare samples simultaneously cultured in the presence of IL-15 vs. cultures in the presence of IL-2, IL-7, gliadin, medium alone or IL-15+M3MoAb. Student's t test for independent samples was used to compare the total number of experiments.

## RESULTS

### **IL-15 induces migration and activation of T cells in treated celiac and control intestine**

The inventors tested in an organ culture model<sup>10,11</sup> to investigate whether challenging biopsies of treated CD with IL-15 induced the migration of CD3+ cells both into the subepithelial (SE) and intraepithelial (IE) mucosal compartments<sup>11</sup>. The T cell distribution was studied in 8 treated CD patients after IL-15 or gliadin challenge, or after challenge with IL-7, IL-2 and IL-4. These cytokines were used as control for IL-15 since they all

share the same  $\beta$  chain receptor and are reported to act in a similar fashion on T cells<sup>13</sup> (Fig 1A). IL-15, but not the other tested cytokines, was as effective as gliadin in modulating SE T cell migration as well as IE T cell infiltration (Fig 1A, Fig 2A-B). IL-15, but not gliadin, was also competent in inducing migration of  $\gamma\delta$ + cells to the IEC (Fig 1A). IL-7, but not IL-4 or IL-2, induced a significant although less vigorous, IE infiltration of  $\gamma\delta$ + T cells (Fig 1A).

The effects of IL-15 on T cell migration were not limited to CD patients, since this cytokine induced T cell migration (with exception of  $\gamma\delta$ + T cells) in non-CD control biopsies (Fig. 1B). In non-CD control intestines gliadin was incompetent to induce any T cell migration, as previously reported (Fig 1B)<sup>11</sup>.

Because of the well known activities of IL-15 on T cell activation and surface antigen modulation<sup>9</sup>, the inventors determined whether IL-15 challenge of biopsies from treated CD patients induced expression of intercellular adhesion molecule-1 (ICAM-1) and IL-2 receptor on lamina propria CD3+ cells. A significant proportion of CD3+ cells was influenced by IL-15 (Fig 3A), and similar results were observed after incubation with gliadin alone, as previously reported<sup>11</sup> (Fig 3A). Even in this case no effect was observed after incubation of treated CD biopsies with IL-7, IL-2 or IL-4 (Fig 3A). IL-15, but not gliadin, induced ICAM-1 and IL-2 receptor expression by lamina propria CD3+ cells also in the intestine of non-CD controls (Fig 3B).

Further studies by the inventors (results not shown) have indicated that IL-15, but not IL-7, IL-4 or IL-2 induced an intraepithelial increase in CD8+ cells in celiac and control

intestine. IL-15 also increases the number of intraepithelial CD94+ cells in celiacs only.

IL-7 was not found to increase CD94+ cells in celiac biopsies.

Gliadin has been demonstrated (results not shown) to induce epithelial migration of CD3+, CD8+ ( $p < 0.001$ ) and CD94+ ( $p < 0.05$ ) cells in celiacs but not in controls.

**IL-15 challenge induces epithelial changes in treated celiac but not in control intestine**

To analyse the mitogenic activity of IL-15 on epithelial cells, the inventors studied the expression of Ki67, one of the earliest markers of cell proliferation<sup>14</sup>. In treated CD biopsies (n=10) IL-15 challenge induced the expression of Ki67 by crypt epithelial cells was induced by IL-15 challenge [percentage of stained cells/100 crypt enterocytes, mean (SD), 8.5(5.7),  $p = 0.007$  vs cultures with medium alone (n=10), mean (SD) 1.5(2.1)]. This effect was not observed in the 5 tested cases of treated CD biopsies after incubation with IL-7 [3.2(1.7)], IL-4 [2.5 (2.6)], IL-2 [5.2(4.9)], nor after IL-15 challenge of all 8 tested non-CD control biopsies (data not shown). In 4 out of 10 treated CD biopsies a few Ki67+ enterocytes were also detected in villus after IL-15 treatment. No Ki67 expression by crypt enterocytes is observed after a short challenge (24h) with gliadin (n=6) [2 (2.6)], as previously reported<sup>11</sup>.

Expression of Ki67 in crypt enterocytes is a characteristic feature of untreated CD intestine and correlates with mucosal damage (Maiuri L. et al., submitted).

The inventors have recently observed that FAS is over-expressed by small intestine epithelial cells of untreated celiac patients (Maiuri L et al., submitted). In view of the epithelial modifications induced by IL-15, the inventors determined whether FAS was also up-regulated on epithelial cells by IL-15. After IL-15 challenge, the expression of FAS was intense (2+) in 7 out of 11 samples and low in 4/11; whilst FAS expression was low to undetectable in 12 out of 14 samples cultured with sole medium (Table 1, Fig 4A-B). Similarly, gliadin challenge was effective in enhancing epithelial expression of FAS in 8 out of 14 tested samples (Table 1), in agreement with our previous report (Maiuri L et al, submitted). whilst no up-regulation of FAS expression was detected after incubation with IL-7, IL-4 or IL-2 (Table 1). IL-15 challenge did not induce expression of FAS in the enterocytes of non-CD controls (low or undetectable FAS expression in all 8 tested cases after culture with IL-15).

Untreated CD patients also over-express FAS-L on their small intestine epithelial cells (Maiuri L et al, unpublished data). The inventors consequently verified whether IL-15 could also induce surface expression of FAS-L on intestinal epithelial cells (Table 1). Biopsies obtained from 5 treated CD patients were studied, in 3 of these cases FAS-L was not observed after 24h culture with medium alone, whilst in two cases FAS-L expression was elevated. In two of the 3 biopsies that remained FAS-L negative after 24 hours incubation with medium, IL-15 induced an increase of the expression of FAS-L. On the contrary, in all these three negative biopsies 24 h of *in vitro* challenge gliadin could not induce an increase in FAS-L expression (Table 1). In none of the 5 tested non-CD control biopsies the expression of FAS-L was increased after culture in presence of IL-15 or medium alone.

**IL-15 induces production of antiendomysium antibodies in treated celiac but not in control intestine**

Since the inventors have recently described that antiendomysium antibodies (EMA) are induced upon *in vitro* gliadin challenge<sup>15</sup>, the inventors also tested the effects of IL-15 on the production of these autoantibodies. They observed that IL-15 was effective in inducing production of EMA in 4 out of 7 tested treated CD biopsies (Table 2). On the contrary, no other cytokine tested. IL-7, IL-2 or IL-4, induced any variation in the EMA levels. Similarly to IL-15, only a challenge with gliadin was able to increase the production of EMA in two out of 4 tested samples, as we previously reported<sup>15</sup>. IL-15, as gliadin, did not induce EMA in any of the 5 tested non-CD control cases.

**Anti-IL-15 neutralising antibodies control the epithelial changes and the production of EMA induced by IL-15 as well as by gliadin challenge in treated celiac intestine**

The results described indicated that IL-15 might play a pivotal role in CD. The key test would be, however, to induce an abrogation of the alternations observed in CD by using anti-IL-15 neutralising MoAbs (monoclonal antibodies). Two MoAbs (M110 and M111) that share the same neutralising characteristics were used for this experiment. In 3 out of 4 treated CD intestine biopsies anti-IL-15 MoAbs were effective in controlling the modifications caused by IL-15, such as expression of FAS, Ki67, as well as EMA production. In 2 out of 3 tested samples anti-IL-15 MoAbs prevented the epithelial expression of FAS and the production of EMA induced by gliadin challenge. Finally in the two samples where FAS-L expression was already intense after incubation with the medium alone, M110 MoAb was effective in down regulating enterocytes expression (Fig

5A-B). On the contrary, neutralising monoclonal antibodies were not effective in controlling the gliadin-induced migration of CD3+ cells into the IEC, as well as the gliadin-induced T cell activation. Anti-human lactase MoAb mlac1, used as isotype control, was devoid of any in vitro effect.

**Anti-IL-15 Neutralising antibodies control gliadin-induced apoptosis of enterocytes in untreated celiac intestine.**

The inventors have found that a 24 h of *in vitro* with gliadin challenge in untreated CD intestine biopsies is effective in producing enterocytes's DNA fragmentation (Maiuri L. et al, submitted). In 3 out of 4 tested cases M110 MoAb was effective in preventing the increase in the number of TUNEL+ enterocytes induced by gliadin [mean (SD) 46(2.6) after gliadin challenge, vs 27.2(7.5) after IL-15 treatment,  $p < 0.05$  (Fig 6 A-B)].

**Expression of IL-15 by lamina propria mononuclear cells (LPMNC) of celiac and control intestines**

To further support the role of IL-15 in the pathogenesis of CD, The inventors determined by immunoistochemistry, the number of LPMNC expressing IL-15, in patients and controls. In untreated celiac patients (14 cases) a significant increase of IL-15+ cells was detected compared to the intestine from controls (8 cases) ( $p < 0.005$ ) (Table 3). Upon gluten free diet (10 cases) the number of IL-15+ cells returned to the normal values ( $p > 0.05$  vs controls) (Table 3). Most of the IL-15+ cells were CD68+, and not surprisingly none was CD3+.



**Effect of IL-15 on CACO-2 cell lines**

Although all the results previously described strongly indicated that IL-15 was causing all the pathognomonic features of CD the inventors could not exclude that some of the modifications, particularly the ones observed on epithelial cells, were not directly induced by IL-15. The inventors therefore challenged with IL-15 the CACO-2 epithelial cell line, which is considered to be a faithful prototype of human intestinal epithelial cells 12, 16, 17. The CACO-2 cells were tested at different stages of *in vitro* maturation.

**IL-15 and IL-2, but not IL-7 nor gliadin induces Ki67 antigen expression.**

Induction of Ki67 is observed in small intestine epithelial cells of untreated CD patients (Maiuri L et al, submitted), as well as previously shown upon *in vitro* IL-15 challenge of small intestine biopsies. The inventors thus analysed the expression of this marker of proliferation on CACO-2 cells challenged with different cytokines. An intense expression of Ki67 was detected in all three experiments in the large majority of the cells (more than 70% of cells/mm<sup>2</sup>) after incubation with IL-15 (Fig 7A) or IL-2 (Fig 7B), whilst in the same experiments Ki67 expression was restricted to a lower number of cells (less than 30% of cells/mm<sup>2</sup>) after incubation with medium alone, IL-7 or gliadin.

**IL-15 but not IL-2, IL-7 nor gliadin induces FAS and FAS-L expression**

As previously indicated, expression of FAS and FAS-L by epithelial cells is a newly defined (Maiuri et al submitted) feature of CD. In 4/6 experiments FAS expression was induced on cell surface after IL-15 challenge (Fig 7C), while it was low after incubation with medium alone, IL-2 (Fig 7D), IL-7 or gliadin (Table 4). Similarly, IL-15 induced

expression of FAS-L in all 4 experiments (Fig 7E), compared to the lower expression observed after incubation with medium alone, IL-2 (Fig 7F), IL-7 or gliadin (Table 4).

#### **IL-15 but not IL-2, IL-7 nor gliadin induces cell death**

Since IL-15 was able to induce the co-expression of FAS and FAS-L on CACO-2 cells the inventors monitored if this event could initiate a suicide/fratricide outcome. After incubation with IL-15 the number of Trypan-Blue positive cells in culture supernatants was higher than mean+3SD of the values observed after incubation with medium alone in 15/16 experiments. The values were significantly higher after IL-15 {(n=16) mean of Trypan-Blue+ cells  $\times 2 \times 10^4/\text{ml}$ , 50.2, SD 41.7, median 36.75, range 7.5-173.5} than after incubation with medium alone (n=7, p=0.0008), IL-2 (n=7, p= 0.007), IL-7 (n=7, p=0.001) or gliadin (n=7, p=0.02). In the experiments in which CACO-2 cells were simultaneously tested with IL-15 vs IL-2 (n=7), or IL-7 (n=7), or gliadin (n=7) or medium alone (n=7), the number of Trypan-Blue+ cells in culture supernatants was significantly higher after IL-15 incubation then after incubation with IL-2 (p = 0.05), IL-7 (p=0.05), gliadin (p=0.02) or medium alone (p=0.01) (Fig 8).

In order to identify whether or not IL-15-induced cell death was mediated by FAS engagement the inventors performed experiments where IL-15 was mixed to neutralising anti-FAS M3 MoAb. In 5 tested cultures the number of Trypan-Blue+ cells in culture supernatants was significantly lower after incubation with IL-15 supplemented with M3 MoAb than after incubation with IL-15 alone (p = 0.01) (Fig 8). After incubation with IL-15 detection of apoptosis by annexin-V and propidium iodide revealed in 4/6 experiments that more than 20% of cells showed signs of apoptosis: positive nuclei

(orange colour) together with surface staining (green colour) (Fig 9A, 9B) in some of them, and only green colour in others. On the contrary, in 4/6 experiments less than 5% of cells became stained after incubation with medium alone or other tested cytokines.

## DISCUSSION

CD has always been considered as the prototype of an immuno-mediated disease in which a single antigen, gliadin, induces T cell activation leading to disease<sup>1,2</sup>. Several aspects of this pathology have pointed in this direction. The first is the strong HLA association, in particular with the heterodimer DQA1\*0501, DQB1\*0201<sup>18</sup>, which has been considered to be the element of genetic restriction for T cells recognising the triggering antigen (gliadin)<sup>2,7</sup>. The second is being the presence of a massive T lymphocyte infiltration of the small intestine with a dramatic increase of intraepithelial lymphocytes<sup>1</sup>. Thus, much of the studies performed in this pathology have been directed towards the dissection of the role and function of T cells<sup>7,19</sup> (Maiuri et al submitted) and related cytokines<sup>20-22</sup> (Maiuri et al submitted). There were, however, signs that the simple T cell immunological recognition of gliadin could not explain all the pathogenic steps of this disease. The first is the unusually high incidence of self-autoantibodies: the EMA in CD<sup>3-6</sup> with the evidence of their synthesis, after gliadin challenge, at the mucosal site<sup>15, 23</sup>. The autoantigen recognised by EMA has been recently defined as tissue transglutaminase<sup>24</sup>. It was also difficult to explain how gliadin could induce the migration of T lymphocytes. Finally, it has been so far impossible to define how T cells could induce the mucosal damage. The inventors have reported that small intestine enterocytes co-express FAS and FAS-L on their surface (Maiuri, et al., submitted), and that the epithelial cells show clear signs of apoptosis<sup>25</sup> (Maiuri, et al submitted). They also observed that gliadin rapidly induces FAS on these epithelial cells, in a way that apparently did not require T cell activation (Maiuri, et al. submitted).

The inventors have demonstrated that a single cytokine, IL-15, can reproduce almost all the features of celiac disease, and more significantly that the modifications induced by gliadin could be, by enlarge, controlled with neutralising anti-IL-15 monoclonal antibodies. These results indicate that IL-15, likely induced at the mucosal level by gliadin, has a central role in the pathogenesis of CD in 3 different ways. Firstly by engaging T cells, secondly, by inducing EMA and thirdly by directly affecting epithelial cell function. Why IL-15 should have such a dominant role? It has already been reported that this cytokine might have a fundamental part in other immuno-mediated diseases such as Rheumatoid Arthritis, by favouring T cell migration<sup>8</sup> and in 'activating' T cells<sup>9</sup>. Even in this study, only IL-15 and not the other cytokines (with the partial exception of IL-7 on  $\gamma\delta$ , induced T cell migration although the effects of IL-15 were not selective for CD patients. This indicates that the activity of IL-15 on T cell migration is not disease restricted. However IL-15 has another unique characteristic, which further suggests a potential role in CD: the ability to directly modulate small intestine epithelial cells<sup>12</sup>. Using the inventors' organ culture model they could demonstrate that small intestine epithelial cells over-expressed transferrin receptor after IL-15 challenge (data not shown), though other two cytokines, IL-4 and IL-2, were also able to modulate this receptor. This is not surprising since it has been reported that intestine epithelial cells express the receptor and respond to IL-2<sup>16, 17</sup>, although very high doses of IL-2 are needed to induce epithelial cell responses<sup>12</sup>. Only IL-15 however was able to up-regulate Ki67 suggesting a trophic action of IL-15, and unexpectedly to consistently induce FAS expression and, although less dramatically, FAS-L upregulation. This is important since in the small intestine of untreated CD patients these markers are over-expressed and the engagement of FAS by its ligand initiate the apoptosis process (Maiuri et al submitted). Moreover, a 24

hour challenge with IL-15 of 4 biopsies from untreated celiacs induced a statistically significant increase of apoptotic epithelial cells compared to cultures incubated with medium alone (data not shown). These findings, induction of FAS, FAS-L and initiation of apoptosis by IL-15 on epithelial cells was completely supported by the studies performed with the CACO-2 cell line, demonstrating that IL-15 directly influenced intestinal epithelial cell. In this context it has to be mentioned that the inventors' results differ from a recent report in which IL-15 was shown to protect T, B cells and hepatocytes from FAS induced apoptosis<sup>26</sup>. The reasons for such an apparent discrepancy might lie in the different experimental procedures, the fact that different species were studied, in our case human and not mouse cells, different target cells and more important, we relate our findings to a well-defined pathology. That IL-15 has a central role in CD is further supported by the compelling evidence that EMA, the most specific marker of CD, are directly induced by this cytokine, and that the gliadin induced production of EMA is blocked by neutralising anti-IL-15 monoclonal antibody. In this context it is of interest, the finding that the spontaneous release of EMA in culture, observed in the supernatant of one treated CD biopsy, was controlled by neutralising anti-IL-15 monoclonal antibodies.

IL-15 could control the induction of EMA by different, and not conflicting, ways for instance by directly influencing B cells<sup>27</sup> as well as acting on T cells<sup>28, 29</sup> or by unmasking the EMA antigen (tissue transglutaminase). Indeed, tissue transglutaminase the autoantigen recognised by EMA<sup>24</sup>, is normally up-regulated in epithelial cells undergoing apoptosis<sup>30</sup>. Thus a scenario might be envisaged in which IL-15 could control the induction of EMA by unmasking the autoantigen, via the autoantigen, via the induction of small intestine epithelial apoptosis, thus promoting the expression of tissue

transglutaminase. In this scheme IL-15 fulfils the role of an agent unmasking a 'hidden' autoantigen (transglutaminase). IL-15 may further influence the production of EMA acting as a locally available growth and differentiation factor for T and B cells<sup>27, 28, 29</sup>.

From our analysis it is apparent that the cells producing IL-15 are, not surprisingly, mononuclear cells in the subepithelial compartment, since T cell are not able to produce IL-15<sup>31</sup>. Epithelial cells seem to be not involved, although small intestine epithelial cells have been shown to produce IL-15<sup>12</sup>. It remains to be clarified how gliadin induces IL-15 and why only epithelial cells of CD patients, as well as CACO-2 cells, are sensitive to the action of this cytokine. These results therefore suggest that two bottle-necks control the induction of CD. The first is the restricted ability of monocytic cells of celiacs to produce IL-15 after gliadin challenge. The second being the specific effects of IL-15 on epithelial cells of celiacs thus, by inducing proliferation, as defined by the expression of Ki67, and ultimately death by allowing the induction of FAS and likely FAS-L. In conclusion we have provided powerful evidence that IL-15 has a central role in CD by directly influencing T cells, inducing EMA production and controlling epithelial damage. In the final analysis the inventors' study provides a totally novel interpretation of the pathogenic mechanisms governing the evolution of one of the most common diseases, provides novel therapeutic targets, and sheds light to define the possible genes involved in this pathology.



Table 1: Expression of FAS and FAS-L by enterocytes after in vitro culture of treated

CD intestine		
Expression of FAS		
Medium Medium + IL-15#° Medium + IL-7# Medium + IL-4# Medium + IL-2# Gliadin °°	Low	Intense
	12/14*	2/14
	4/11	7/11
	3/3	0/3
	3/3	0/3
	3/3	0/3
	6/14**	8/14
Expression of FAS-L		
Medium Medium + IL-15 Gliadin	Low	Intense
	3/5	2/5
	1/5	4/5
	3/5	2/5

\* undetectable in 5 cases, \*\* undetectable in 4 cases.  
# in all cases low expression of FAS after incubation with medium alone.  
° p = 0.014 vs medium alone  
°° p = 0.023 vs medium alone

Table 2: Production of EMA in cultured treated CD intestinal explants

	EMA absent		EMA present
Medium	7/7		0/7
medium + IL-15*	3/7		4/7
medium + IL-7	3/3		0/3
medium + IL-2	3/3		0/3
medium + IL-4	3/3		0/3
Gliadin	2/4		2/4

in another sample EMA were detected after incubation with medium alone

\*p = 0.034 vs cultures with medium alone.

Table 3: Expression of IL-15 by LPMNC of CD and control intestine

	Controls	Untreated CD with villus atrophy	Treated CD
	n=8	n=14°	n=10°°
Median	4	21.5	4
Range	0-12	4-44	0-12.2
Mean	5	23.5	4.8
SD	4.4	13.5	3.8

Number of IL-15+ cells per mm² of lamina propria

°p<0.005 vs controls and vs treated CD; °°p>0.05 vs controls.

Table 4: Effect of IL-15 on CACO-2 cell line\*

	Medium		Medium+ IL-15		Medium+IL-7		Medium+IL-2		Medium+gliadin	
	low	high	Low	High	Low	High	low	high	low	high
FAS	2/3	1/3	2/6	4/6	3/3	0/3	2/2	0/2	3/3	0/3
FAS-L	3/4	1/4	0/4	4/4	2/2	0/2	3/3	0/3	2/2	0/2

\* 24 h incubation of CACO2 cells

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IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels  
A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells  
Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *J Pathol* 179, 288-93 (1996).
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**CLAIMS**

1. An antagonist of Interleukin-15 (IL-15) for treating an inflammatory bowel disease.
2. An antagonist according to claim 1, wherein the inflammatory bowel disease is celiac disease.
3. A method of treating an inflammatory bowel disease comprising the step of administering to a patient a pharmaceutically-effective amount of an antagonist of IL-15.
4. A method, according to claim 3, wherein the inflammatory disease is celiac disease.
5. An antagonist or method, according to any previous claim, wherein the antagonist is a mutein of mature, or native, IL-15.
6. An antagonist or method, according to claim 5, wherein the mutein is a mutation of a simian IL-15 or a human IL-15.
7. An antagonist or method, according to claim 5 or claim 6, wherein the IL-15 antagonist comprises a deletion or substitution mutation with a different naturally-occurring amino acid residue at one or both of Asp 56 and/or Gln 156.



8. An antagonist or method, according to any one of claims 5-7, wherein the mutein is conjugated to a further chemical moiety.
9. An antagonist or method, according to any one of claims 1-4, wherein the antagonist is an antibody or an effective fragment thereof.
10. An antagonist or method, according to claim 9, wherein the antibody is a monoclonal antibody against IL-15.
11. An antagonist or method, according to any one of claims 1-4, wherein the antagonist is an IL-15 molecule that is covalently bonded with a chemical group that interferes with the ability of IL-15 to effect a signal transduction through either the  $\beta$  or  $\gamma$ -subunits of the IL-15 receptor complex but does not interfere with IL-15 binding to IL-15 R $\alpha$ .
12. An antagonist of IL-15 for use in the manufacture of a medicament to treat an inflammatory bowel disease.
13. A suppository comprising an IL-15 antagonist.
14. A tablet for oral administration comprising an IL-15 antagonist in combination with an IL-15 antagonist.

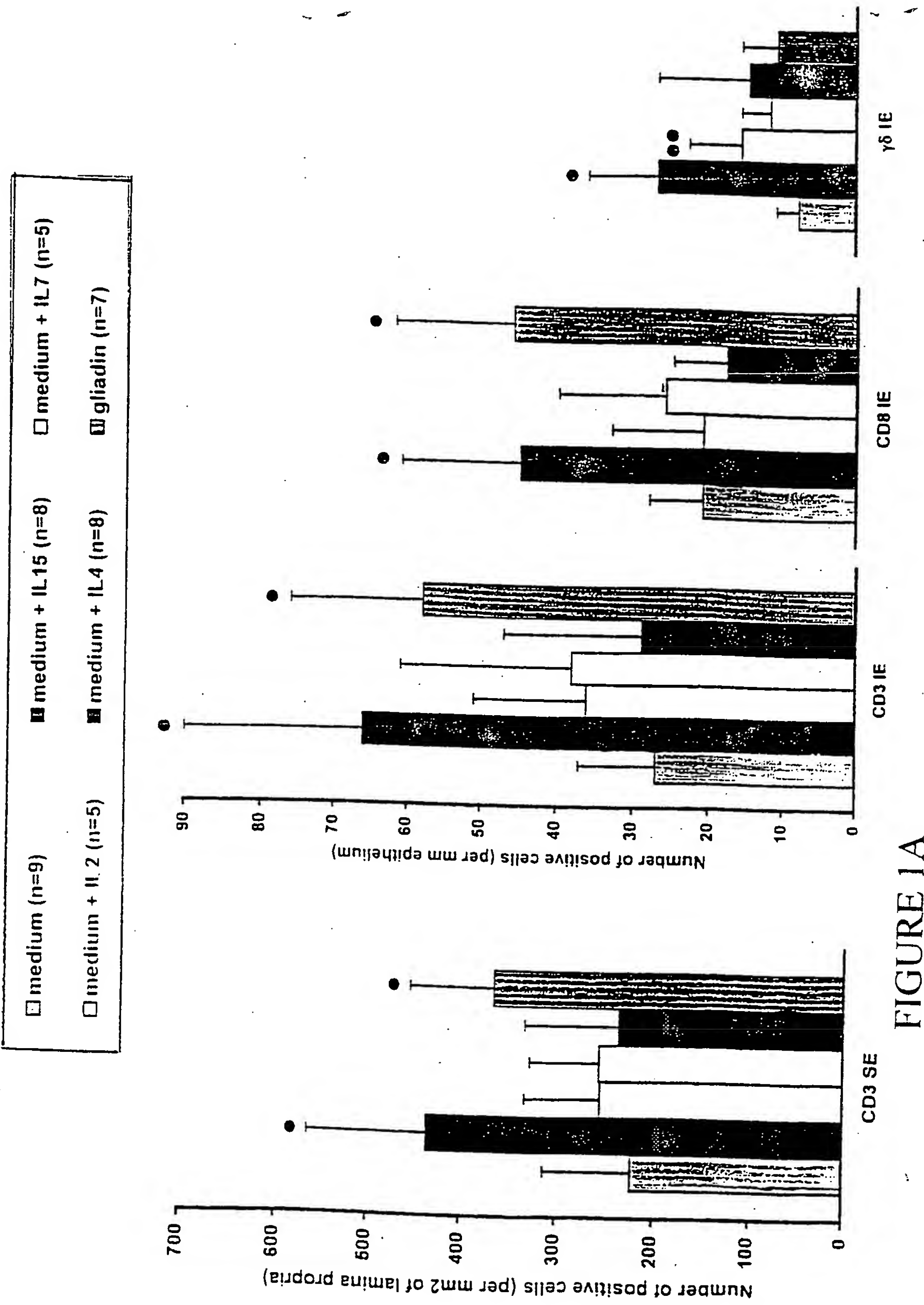


FIGURE 1A

□ medium (n=5)      ■ medium + IL15 (n=5)      ▨ gliadin (n=5)

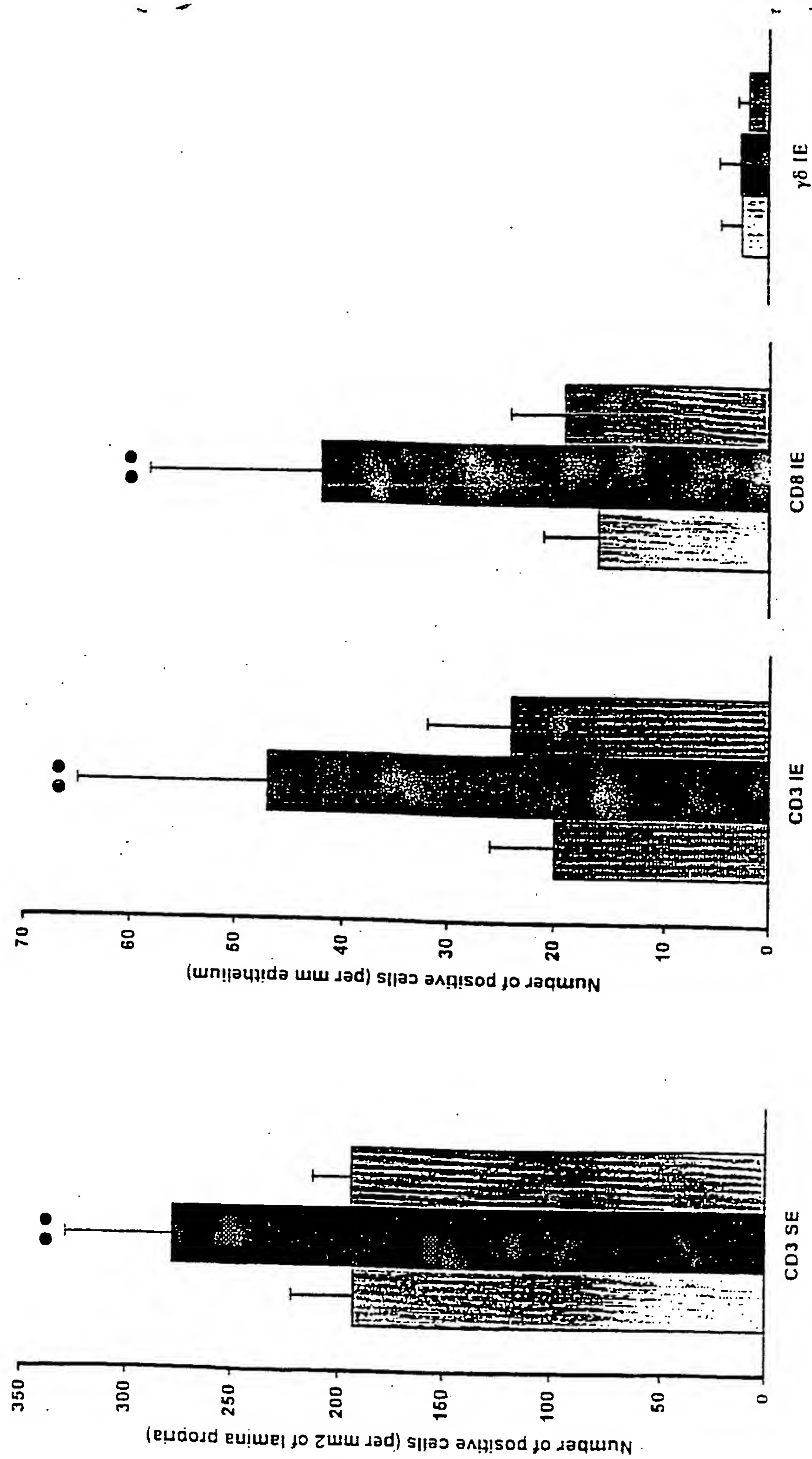


FIGURE 1B



FIGURE 2A



FIGURE 2B

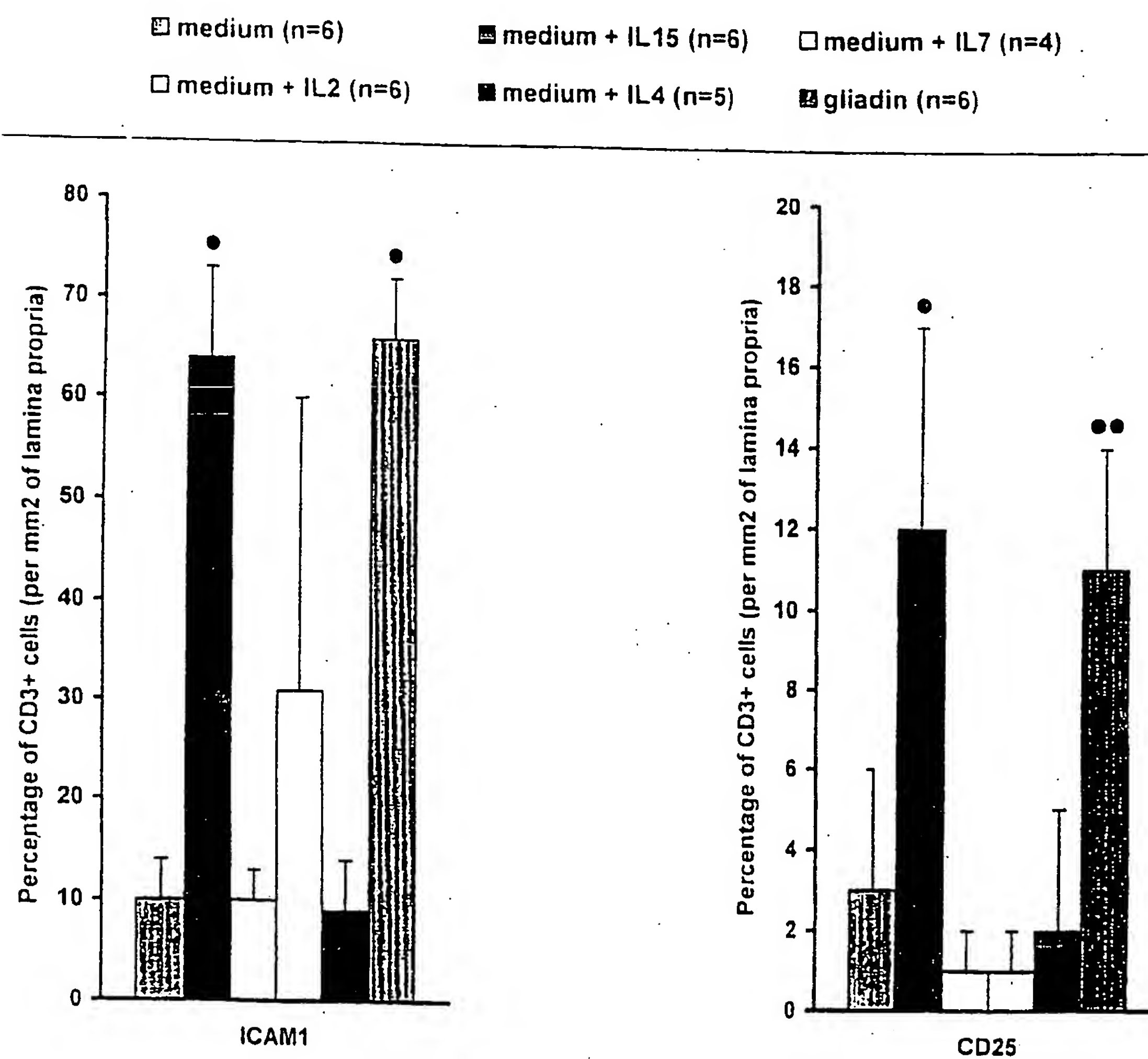


FIGURE 3A

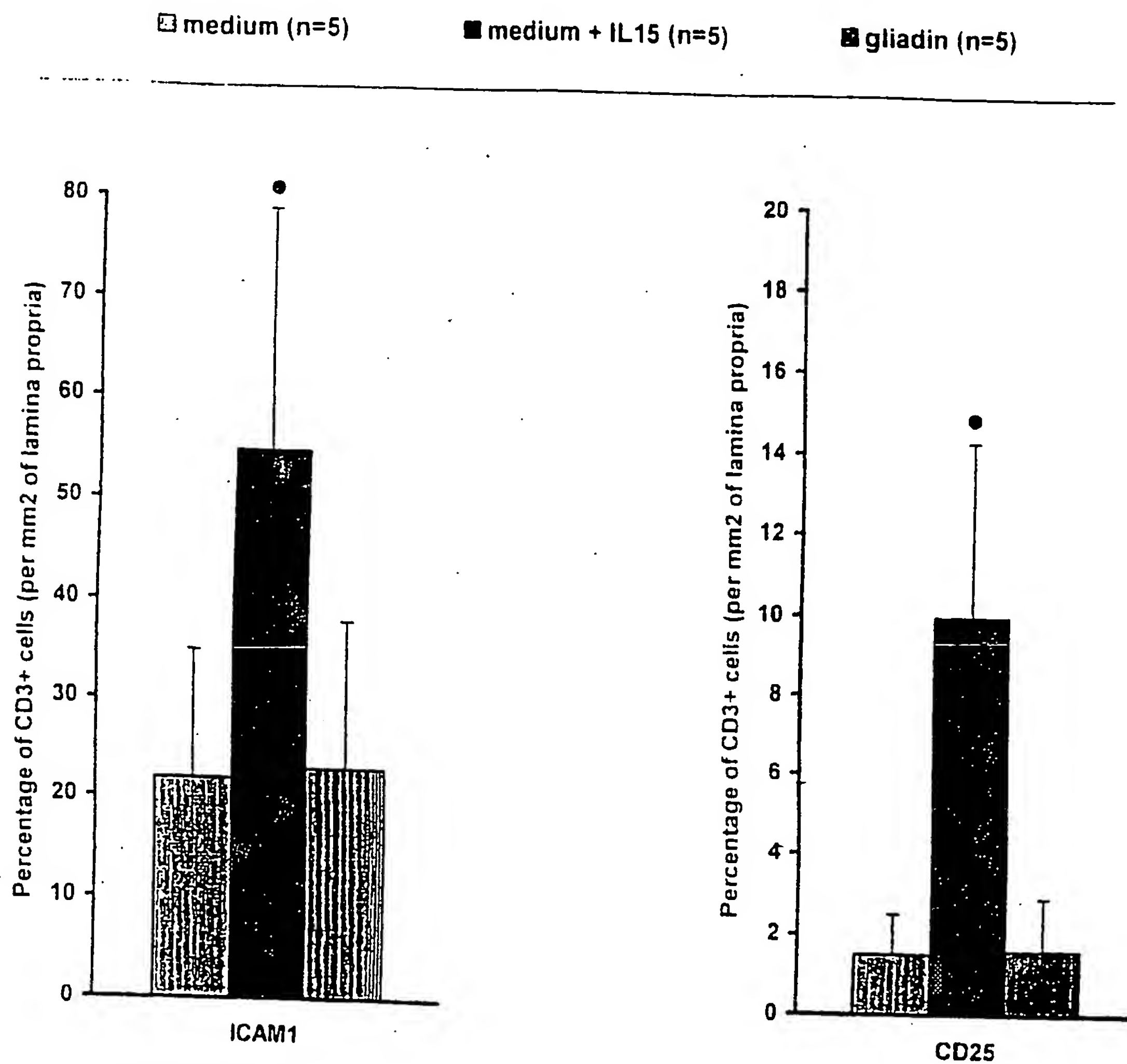


FIGURE 3B



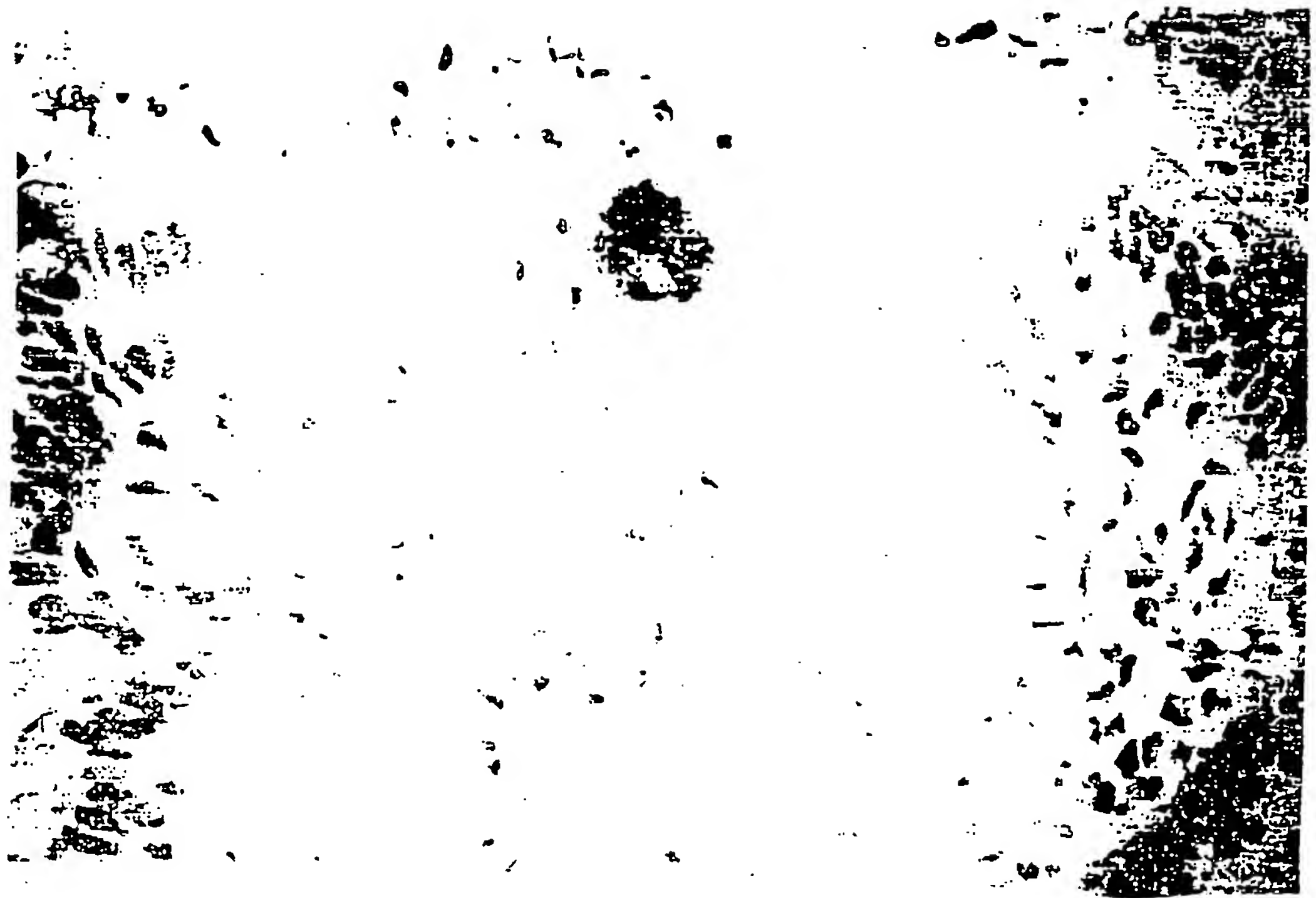


FIGURE 4A



FIGURE 4B



FIGURE 5A



FIGURE 5B

Figure 6A

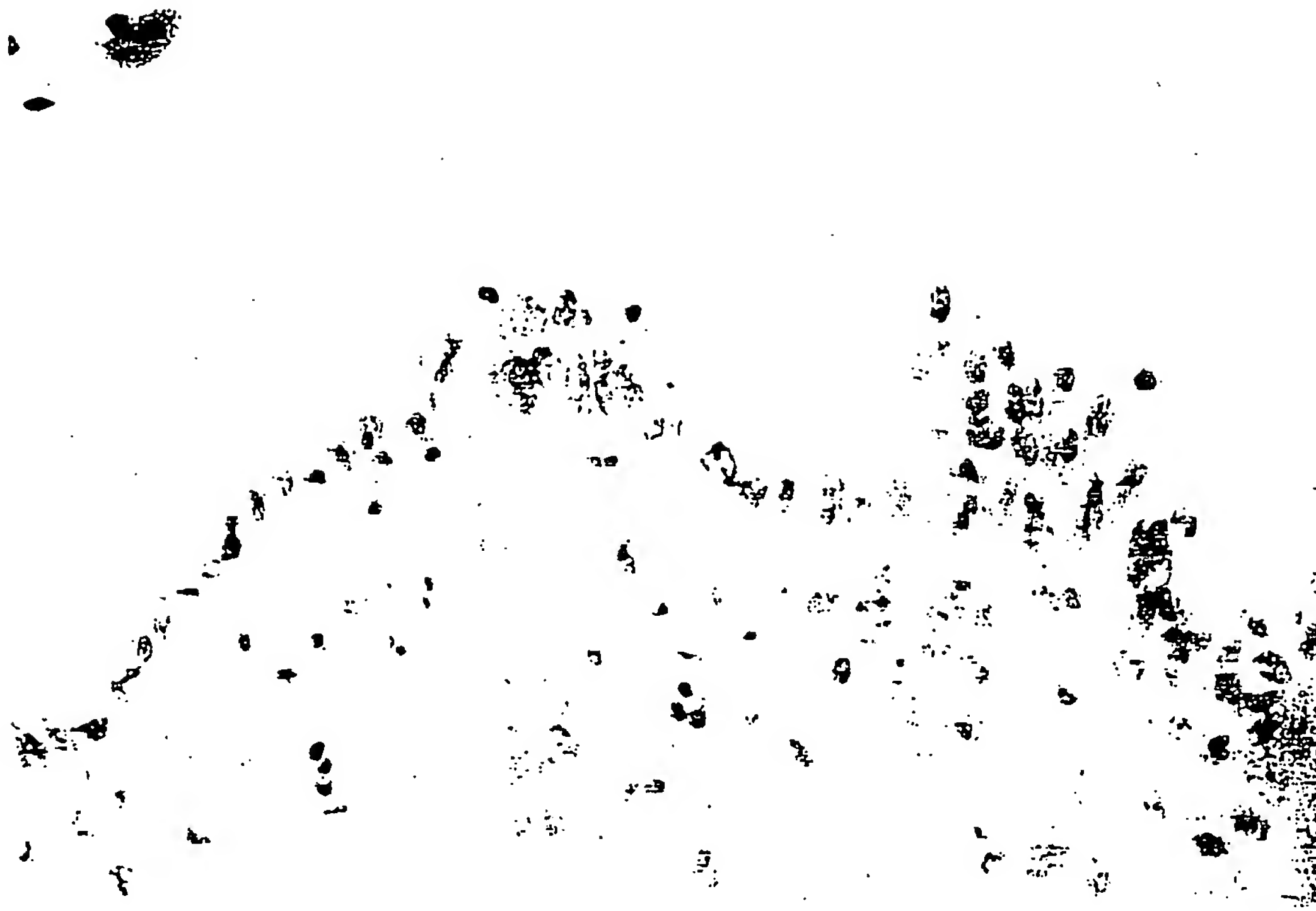


Figure 6B

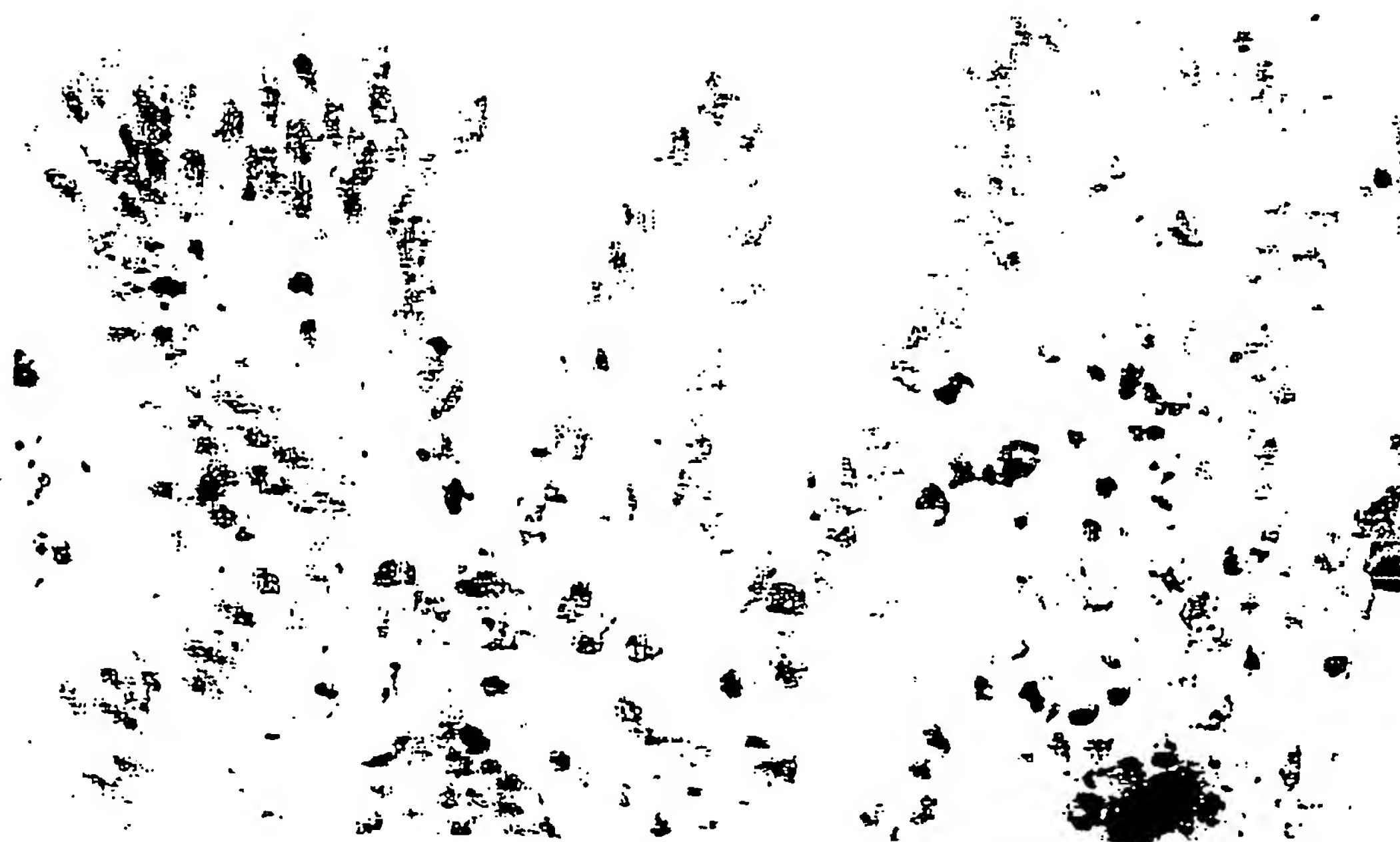




Figure 7A

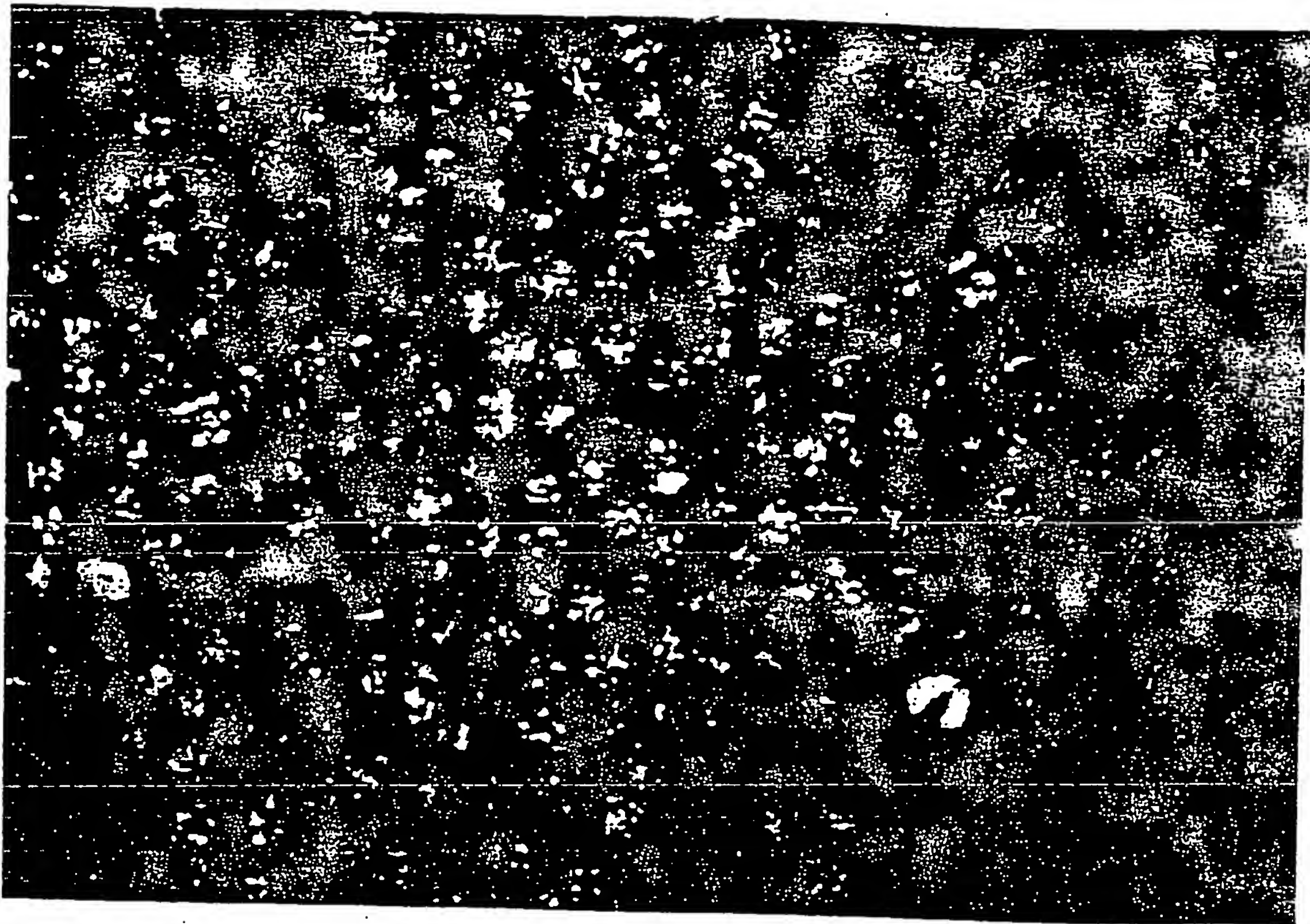
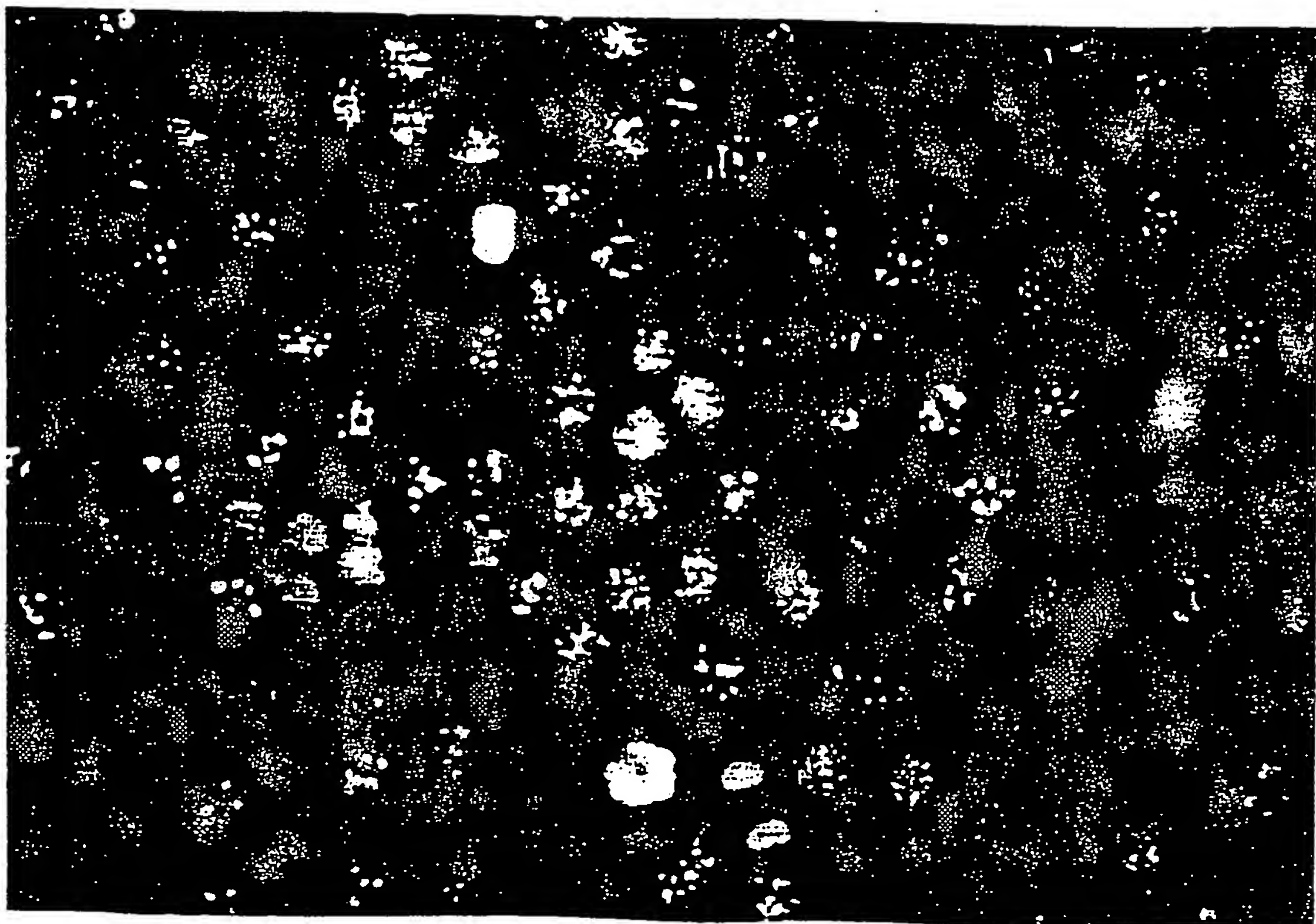


Figure 7B



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Figure 7C

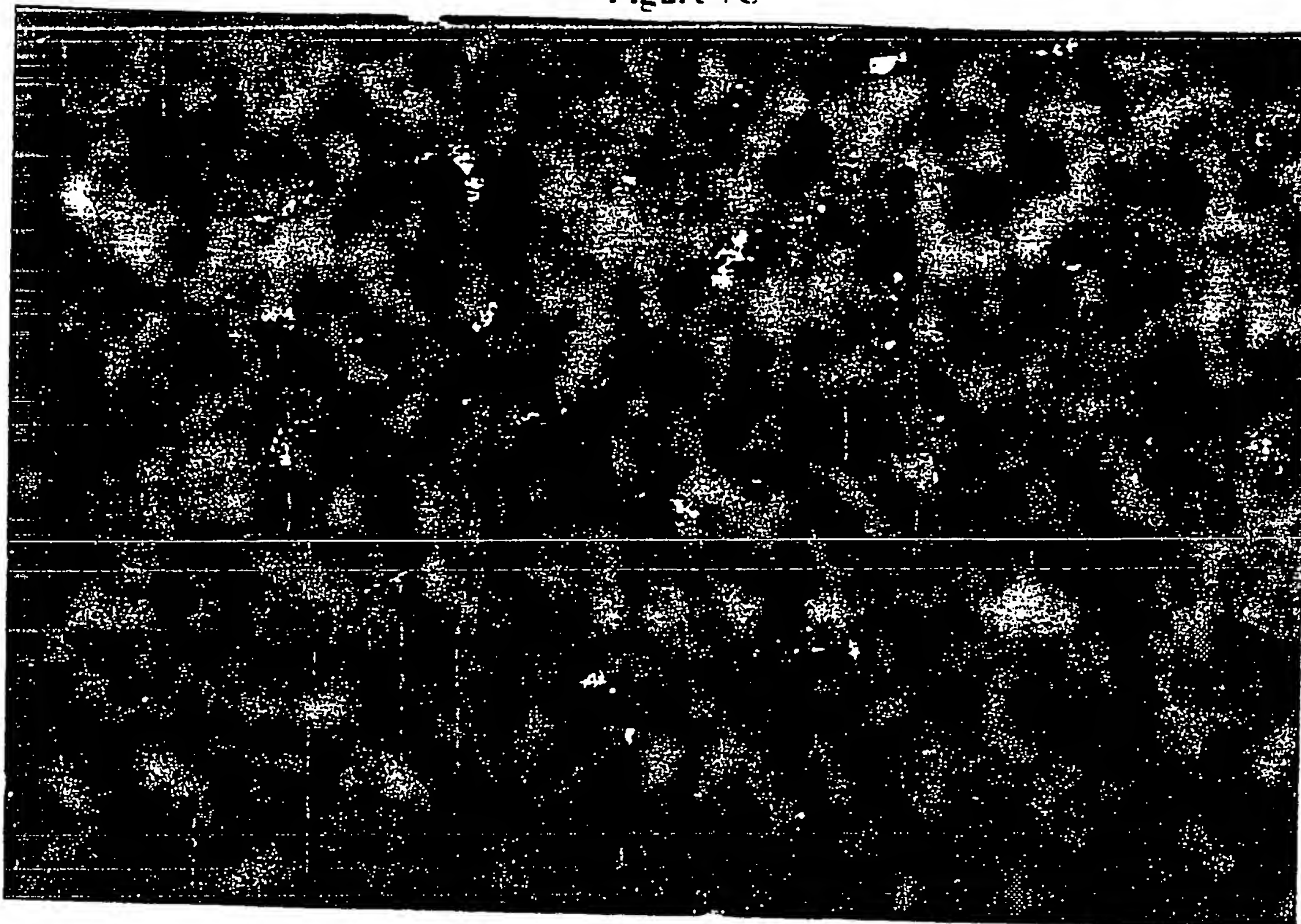


Figure 7D





Figure 7E

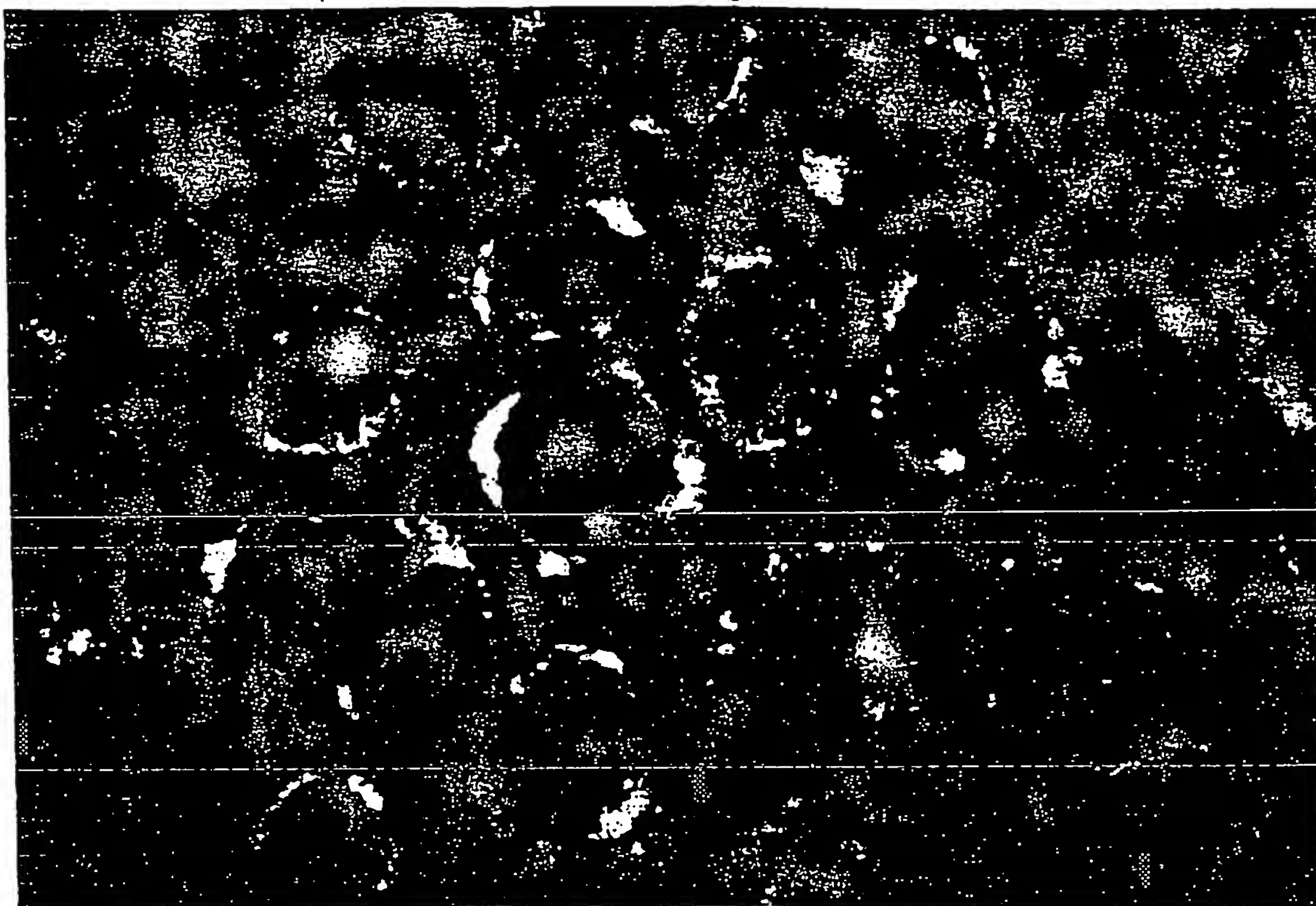
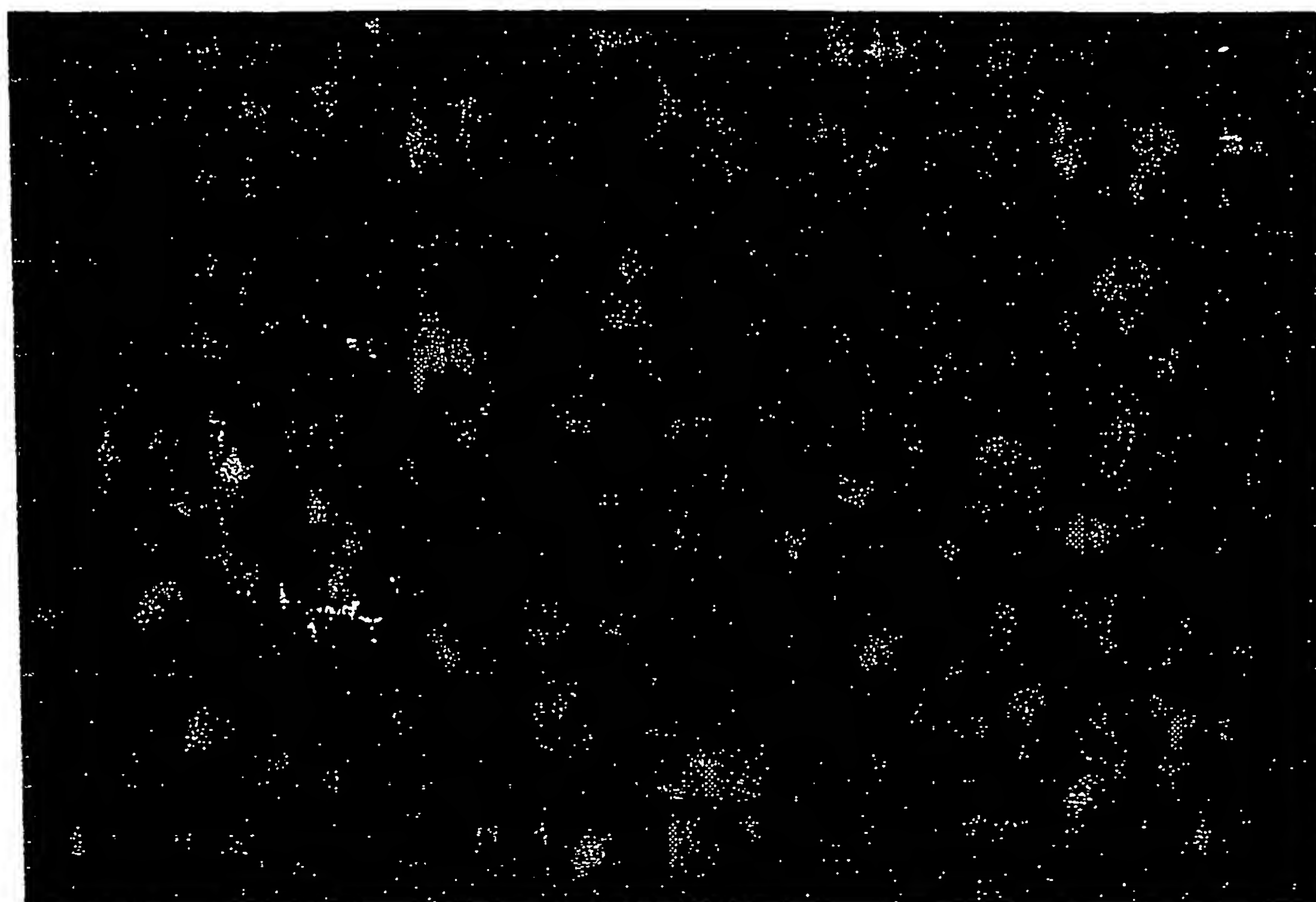


Figure 7F



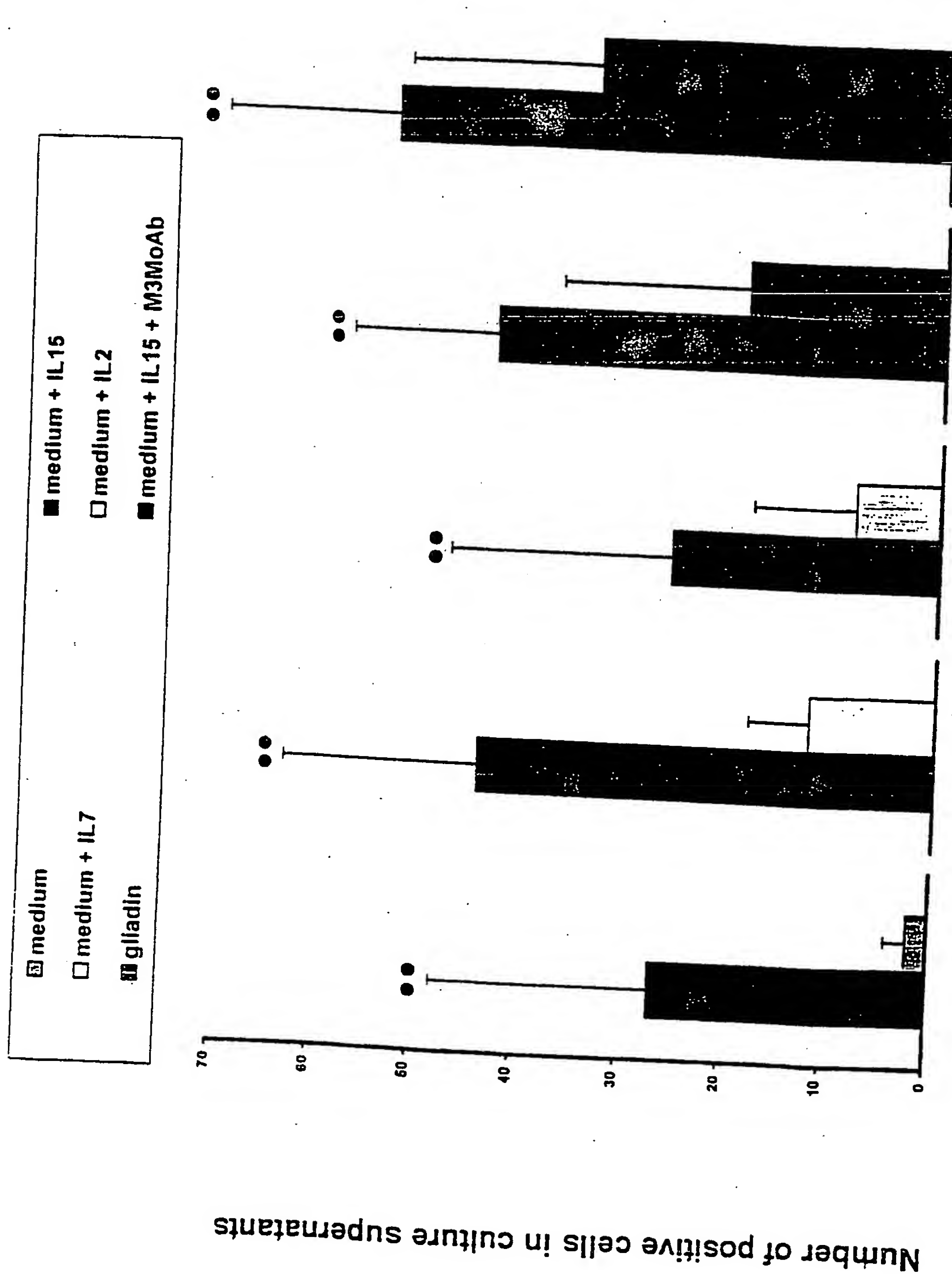


FIGURE 8

Figure 9A

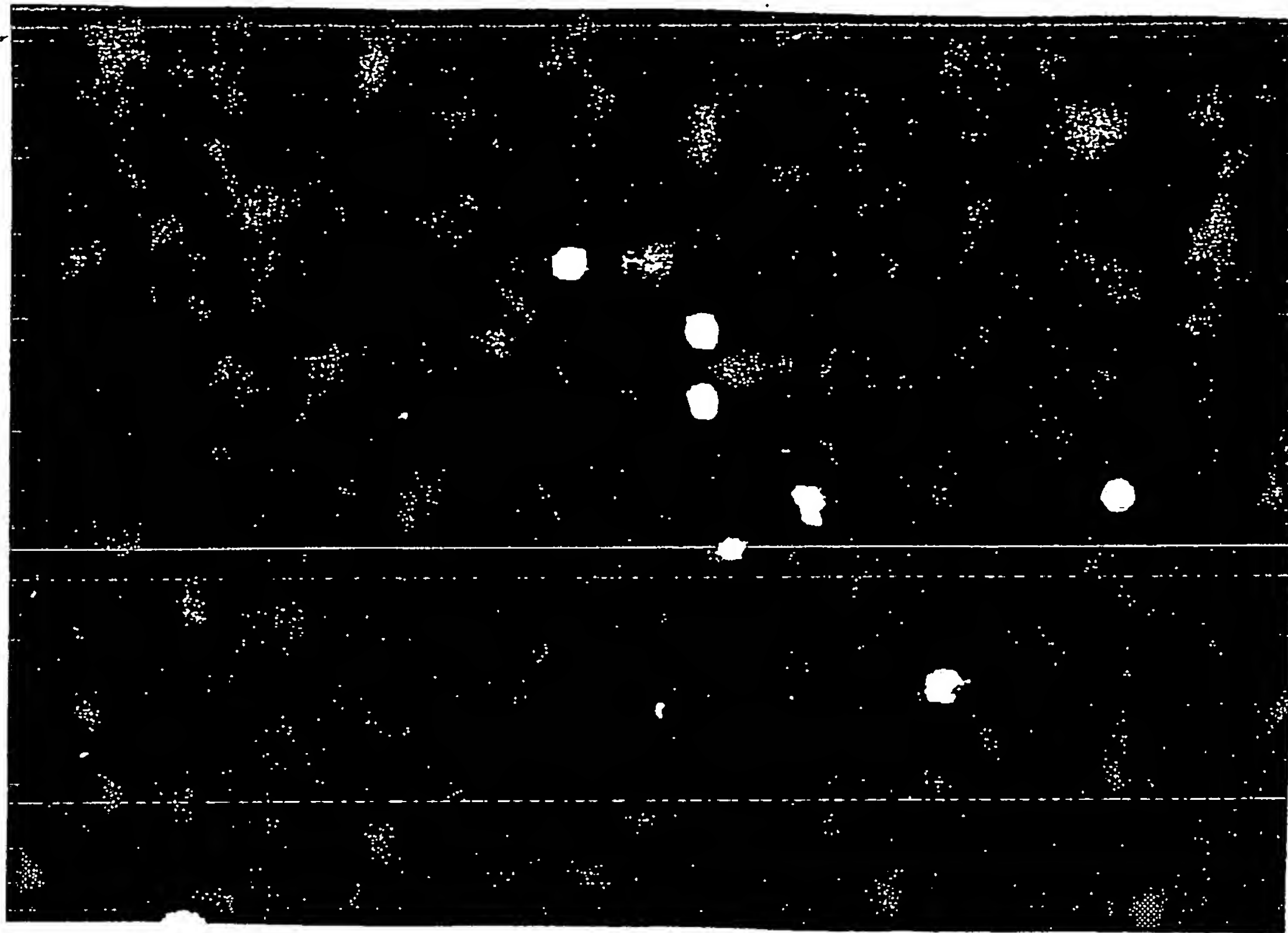
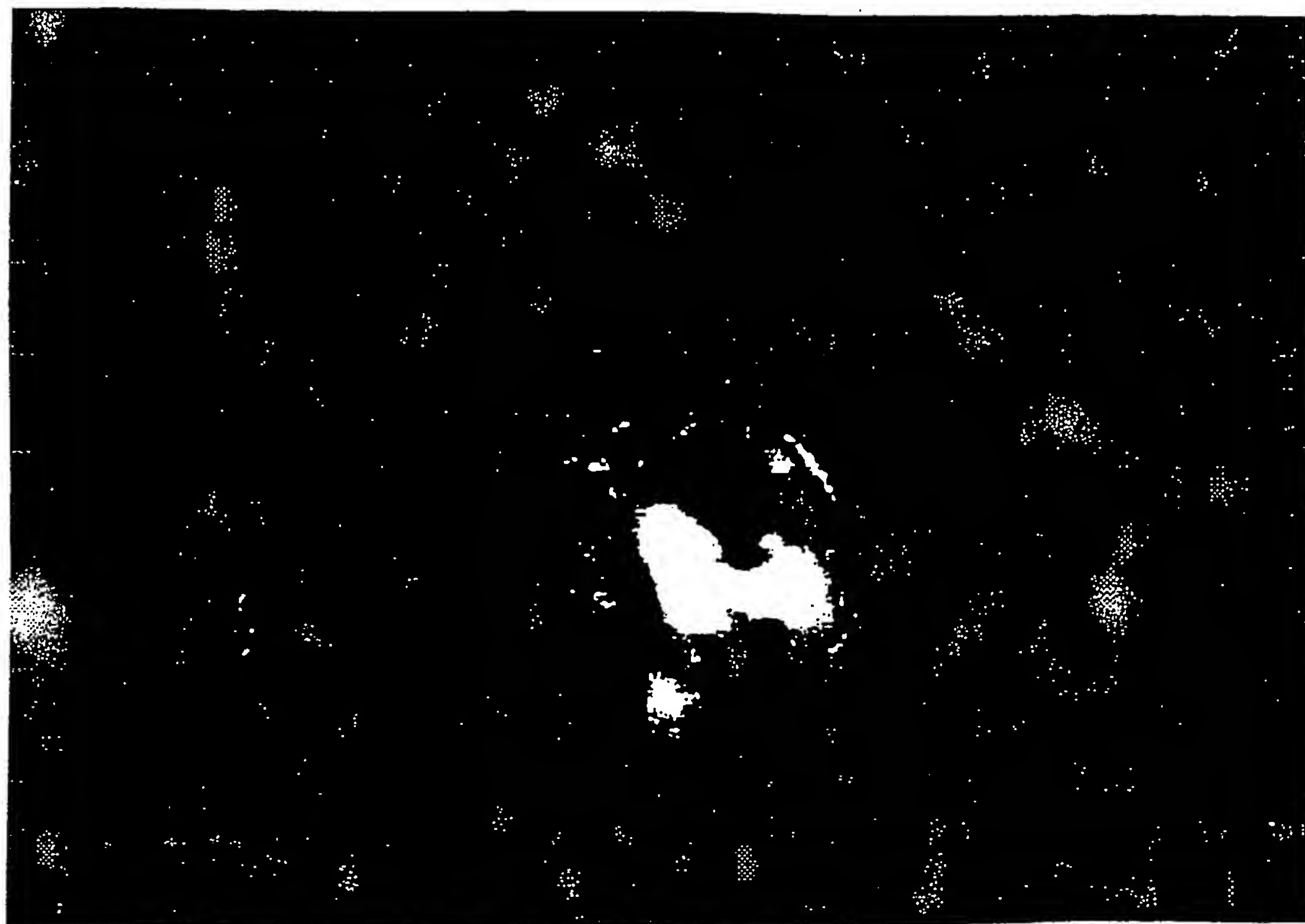


Figure 9B



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/GB99/02201 <b>(22) International Filing Date:</b> 9 July 1999 (09.07.99)  <b>(30) Priority Data:</b> 9814892.7 10 July 1998 (10.07.98) GB  <b>(71) Applicant (for all designated States except US):</b> THE MATHILDA AND TERENCE KENNEDY INSTITUTE OF RHEUMATOLOGY [GB/GB]; 1 Aspenlea Road, Hammersmith, London W6 8LH (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> LONDEI, Marco [IT/GB]; The Mathilda & Terence Kennedy Institute of Rheumatology, 1 Aspenlea Road, Hammersmith, London W6 8LH (GB). QUARATINO, Sonia [IT/GB]; 3 Prebend Mansions, Chiswick High Road, London W4 2LU (GB). MAIURI, Luigi [IT/IT]; Via Gioacchino Toma, 6/c, I-80127 Napoli (IT).  <b>(74) Agents:</b> BANNERMAN, David, Gardner et al.; Withers & Rogers, Goldings House, 2 Hays Lane, London SE1 2HW (GB).		<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 16 March 2000 (16.03.00)
<b>(54) Title:</b> TREATMENT OF CELIAC DISEASE WITH INTERLEUKIN-15 ANTAGONISTS  <b>(57) Abstract</b>  The invention relates to the treatment of inflammatory bowel diseases, such as celiac disease with interleukin-15 (IL-15) antagonists. Preferably the antagonists are muteins of IL-15, antibodies against IL-15 or IL-15 molecules bound to chemical groups that interfere with the ability of IL-15 to effect a signal transduction through either the $\beta$ or $\gamma$ -subunit of the IL-15 receptor complex, but which do not interfere with IL-15 binding to IL-15R $\alpha$ .		



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# INTERNATIONAL SEARCH REPORT

National Application No  
PCT/GB 99/02201

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/20 A61K39/395 A61K47/48 A61K9/02 A61K9/20

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 41232 A (BETH ISRAEL DEACONESS MEDICAL CENTER) 6 November 1997 (1997-11-06) page 10 page 14 claims 1,31	1-8, 12-14
X	WO 96 26274 A (IMMUNEX CORPORATION) 29 August 1996 (1996-08-29) page 29 -page 30	1,2,5-14
X	US 5 707 616 A (GRABSTEIN K.H. ET AL) 13 January 1998 (1998-01-13) column 7 -column 8 column 19, line 55 -column 20, line 22	1-4,9, 10,12-14



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

18 January 2000

Date of mailing of the international search report

25/01/2000

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 99/02201

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 3-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No

PCT/GB 99/02201

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